American Journal of

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CONTENTS

The Incidence of Arthritis in Swine Following Vaccination for Swine Erysipelas..... 5
C. M. Neher, C. B. Swenson, L. P. Doyle, and D. Sikes

The Role of Hormones in the Relaxation of the Uterine Portion of the Cervix in Swine..... 15
J. C. Smith and A. V. Nalbandov

The Use of Piperazines as Anthelmintics for Swine..... 19
F. D. Ensle, E. H. Wilkens, and M. L. Colglazier

Electrophoretic Patterns of Blood Serum from Pig Fetuses and Young Pigs..... 25
L. Rutqvist

Toxicity of Heated Swine Serum..... 32
J. Mathews and D. A. Buthala

Effect of Cortisone on the Mechanism of Anaplasma Immunity in Experimentally Infected Calves. I. Hematological and Immunoserological Studies..... 37
M. Ristic, F. H. White, J. H. Green, and D. A. Sanders

Effect of Cortisone on the Mechanism of Anaplasma Immunity in Experimentally Infected Calves. II. Studies of Pathological Changes..... 44
M. Ristic and W. L. Sippel

Studies of the Bovine Adrenal Gland. II. The Histological and Cytochemical Effects of the Administration of 1, 1 Dichloro-2, 2-Bis (P-Chlorophenyl) Ethane on the Adrenal Cortices of Dairy Calves..... 51
A. F. Weber, J. T. Bell, Jr., and A. F. Sellers

Epithelial Tumors of the Bovine Gallbladder—A Report of Eighteen Cases..... 58
W. A. Anderson, A. W. Moulux, and C. L. Davis

The Cytopathogenesis of Vesicular Stomatitis Virus Infection in Cattle..... 66
W. E. Ribelin

Persistence in Cattle of Serum-Neutralizing Antibodies of Vesicular Stomatitis Virus 74
D. K. Sorensen, T. L. Chow, T. Kowalczyk, R. P. Hanson, and C. A. Brandly

Experimental Mixed Infection of Cattle with Foot-and-Mouth Disease Viruses..... 78
R. G. Cunha, I. Torturella, J. L. Saile, and U. M. Serrao

Distribution of Penicillin in Mastitic Udders Following Intramammary Injection—An Autoradiographic Study..... 84
S. Ullberg, E. Hansson, and H. Funke

The Use of Huddleson's Brucella M Vaccine Under Field Conditions..... 93
N. B. King, B. H. Edgington, and N. A. Frank

The Effects of Prepartum Administration of Progesterone to the Cow..... 97
L. E. McDonald and R. L. Hays

Morphological and Serological Characteristics of Fresh Isolates of *Vibrio Fetus* and Other Vibrios Inhabiting the Bovine and Ovine Genital Tracts..... 99
M. Ristic, F. H. White, and R. B. Doty

(Continued on page 1)

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(Contents Continued from Cover)

A Comparison of the Effect of Catalase-Positive and Catalase-Negative Vibrio on the Fertility of Cattle 108
A. H. Frank, J. H. Bryner, and B. Caruthers

Several Blood Constituents of Five Breeds of Dairy Cattle in Guatemala 112
R. L. Squibb, J. E. Braham, M. A. Guzmán, and N. S. Scrimshaw

Esophageal Innervation and the Eructation Reflex in Sheep 115
R. W. Dougherty, R. E. Habel, and H. E. Bond

Effectiveness of Dow ET-57 (Trolene)* Against the Nasal Botfly of Sheep 129
H. O. Peterson, E. M. Jones, and N. G. Cobbett

A Comparison of Reconstituted Skim Milk and Egg Yolk-Sodium Citrate as Extenders for Ram Semen 132
J. R. Hill, V. Hurst, and W. C. Godley

Distribution of Aqueous Penicillin and Penicillin in Oil in Normal Goat Udders Following Intramammary Injection—An Autoradiographic Study 135
S. Ullberg, E. Hansson, and H. Funke

Experimental Leptospirosis. III. Caprine *Leptospira Pomona* Infection 139
E. V. Morse and R. F. Langham

Prevalence of Agglutinins in Canine Serums to Serotypes Other than *Leptospira Canicola* and *Leptospira Icterohaemorrhagiae*—Report of Isolation of *Leptospira Pomona* from a Dog 145
L. C. Murphy, P. T. Cardeilhac, A. D. Alexander, L. B. Evans, and R. H. Marchwicki

Cytopathology and Rate of Release of Infectious Canine Hepatitis Virus Grown in Dog Kidney Cells 152
R. W. Leader

Hemangiopericytoma in the Dog 159
D. H. Yost and T. C. Jones

Feline Infectious Anemia. I. Clinical Aspects 164
J. C. Flint, M. H. Roepke, and R. Jensen

The Effect of Chlortetracycline Treatment of Turkeys Affected with Ornithosis 169
D. E. Davis and J. P. Delaplane

Propagation of the Lapinized-Avianized Strain of Rinderpest Virus in the Culture of Chicken Embryo Tissue 174
J. Nakamura, T. Motohashi, and S. Kishi

The Pathology of Experimentally Produced Histomoniasis in Turkeys 181
T. D. Malewitz, R. A. Runnells, and M. L. Calhoun

Lesions in the Cerebellum and in the Reticular and Vestibular Centers in Newcastle Disease 186
D. J. Sullivan

Factors Influencing the Growth of Newcastle Disease Virus in Isolated Chorioallantoic Membranes 191
F. Zuschek, R. P. Hanson, and C. A. Brandly

Furazolidone Treatment of Experimental Pullorum Disease in Adult Chickens 196
W. Henderson, F. L. Walkey, and G. L. Morehouse

A Comparison of the Indirect Complement-Fixation Test, the Direct Complement-Fixation Test, and the Macroscopic-Agglutination Test for Ornithosis Antibodies in Turkey Serums 200
J. E. Neal and D. E. Davis

Growth and Regression in Chickens of a Duck Variant of the Rous Sarcoma 204
E. F. Waller, G. H. Warren, and R. Fagan

Infectivity of Colonial Variants of *Vibro Fetus* Strains for the Chicken Embryo 205
F. H. White, M. Ristic, and D. A. Sanders

The Influence of Maturity and Sex on the Blood Pressure of the Turkey 209
H. S. Weiss and M. Sheahan

The Pathology of Aleutian Disease in Mink 212
C. F. Helmboldt and E. L. Jungherr

(Continued on page 3)

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(Contents Continued from Page 1)

Egg-Adaptation of a Strain of Distemper Virus Isolated from a Raccoon..... 223
E. Crook and S. H. McNutt

Detection of Pulmonary Tuberculosis in Laboratory Monkeys by Chest Radiography..... 225
W. C. Dolowy, M. H. Frank, G. E. Cox, and A. L. Hesse

The Effects of Heat and Hemoglobin on the Serum Proteins of the Guinea Pig..... 230
G. T. Dimopoullos and O. N. Fellowes

Primary Isolation and Comparative Titrations of Five Field Strains of Vesicular Stomatitis Virus in Chicken Embryos, Hogs, and Mice..... 233
L. Karstad and R. P. Hanson

Effects of Hypotonic and Hypertonic Sodium Chloride Solutions on First-Instar *Hypoderma Lineatum* Larvae..... 237
P. Gailiunas

A Modified Erlenmeyer Flask for Use in Culturing Entire Yolks of Turkey Eggs..... 239
B. W. Bierer

Bacteriophage Typing of Micrococci of Bovine Origin..... 241
J. T. Seto and J. B. Wilson

The Detection of Isopropyl Alcohol in Frozen Semen..... 247
P. W. Murdick

A New Approach in Chemotherapeutic Trials Against Trichomonas Infections..... 249
R. Rubin and D. R. Cordray

Observations on the Morphology of *Anaplasma Marginale* with Reference to Projections or Tails..... 252
T. E. Franklin and H. E. Redmond

Isolation of an Organism Resembling *Clostridium Carnis* from Mink..... 254
E. M. Gianforte and R. Brown, Jr.

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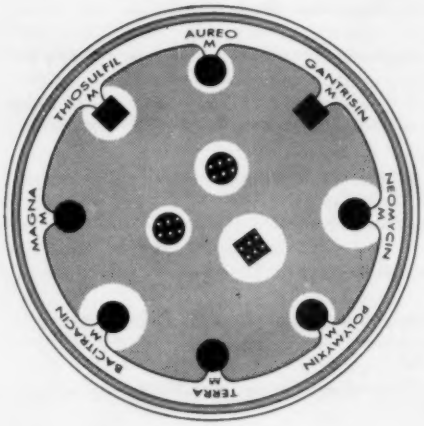

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The Incidence of Arthritis in Swine Following Vaccination for Swine Erysipelas

GEORGE M. NEHER, Ph.D.; CHARLES B. SWENSON, D.V.M.; LEO P. DOYLE, D.V.M., Ph.D.; D. SIKES, D.V.M., Ph.D.

Lafayette, Indiana

ERYSIPELOTHRIX RHUSIOPATHIAE has been clearly incriminated as the cause of swine erysipelas and as a cause of rheumatoid arthritis in swine, which may or may not result as a sequel to the acute infection.[1, 7, 17, 18, 22] Arthritis continues to rank as one of the principal reasons for the condemnation of swine carcasses in federally inspected meat-packing plants in the United States,[11] in spite of the widespread use of procedures for the immunization of swine against erysipelas.

A survey of Erysipelothrix infection in Canada likewise has indicated an increased incidence of arthritis in swine in recent years.[2] Sikes *et al.*[19] recently reported the results of vaccination experiments on 41 swine, some of which were protected with a commercially prepared bacterin. Although the infectivity rate was high when challenged intravenously, some protection against death was afforded by vaccination, *i.e.*, the mortality was 20 per cent in vaccinated and over 50 per cent in unvaccinated swine. Approximately 70 per cent of the vaccinated survivors of the six-week challenge developed chronic arthritis.

These findings warrant confirmation and further investigation, which should include vaccination with other commercially available immunizing products, challenging swine more than 12 weeks after vaccination, and using less severe methods of challenge that may simulate natural conditions, such as scarification of the skin and topical application of culture and contact exposure.

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Submitted as journal paper No. 1104, Agricultural Experiment Station, Lafayette, Ind.

This investigation was supported in part by a research grant, A-45 (C-4), from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U. S. Public Health Service.

MATERIALS AND METHODS

Of 96 swine used in these experiments, 66 were vaccinated against erysipelas, and the remaining 30 were unvaccinated controls. They were purebred Chester White shoats obtained from a drove in which no clinical signs of swine erysipelas or arthritis had been observed since the herd was established in 1941.

All were immunized against hog cholera, using serum and virus simultaneously, between three to six weeks prior to vaccination for erysipelas. They were in excellent health, 3 to 4 months old, and weighed 75 to 120 lb. They were in pasture lots with portable hog houses under semi-isolated conditions. At the time of challenge, they were moved into a barn and kept in pens with concrete floors. Water and a balanced, commercially prepared "hog grower," to which no antibiotics were added, were provided ad libitum.

The 53 vaccinated swine used in the first two experiments were given a commercially prepared erysipelas bacterin of the aluminum hydroxide adsorbed type, according to the recommendations of the manufacturer. Housed with them were 22 unprotected swine which were later challenged as unvaccinated controls. The 13 immunized swine used in the third trial were vaccinated with 1 cc. of a commercially prepared desiccated live culture of *Ery. rhusiopathiae*, administered concomitantly with 20 cc. of anti-swine erysipelas serum. Housed with them were 8 unvaccinated shoats.

The culture of *Ery. rhusiopathiae* used for challenge was originally isolated from a hog that had died of acute septicemic erysipelas. The primary isolation was made on tryptose agar (Difco), and subsequent stock cultures were incubated for 24 hours in a semisolid medium (Difco, brain, liver, and heart, pH 7.2), to which 10 per cent sterile

horse serum had been added. The cultures were maintained by monthly transfers and, after incubation, were kept in a refrigerator. With the exception of the first experiment, three daily transfers were made in semisolid medium prior to the time of challenge. It was believed that this procedure insured that the microorganism would be in a rapidly growing state at the time the exposures were made.

The intravenous challenge consisted of the injection of 10 cc. of a 24-hour culture of *Ery. rhusiopathiae* in semisolid medium into the anterior vena cava. In the swine exposed by scarification, the hair was clipped from an area on the side, roughly 10 inches square, scarification was accomplished by drawing the point of a scalpel over the skin until serum exuded, and 5 cc. of a 24-hour culture of the organisms was applied topically. The spleens, livers, and kidneys of the animals that died were routinely cultured on tryptose agar (Difco) slants.

RESULTS

EXPERIMENT 1

Six-Week Challenge.—In the first experiment, 10 of 20 pigs (4 mo. old, 110 to 120 lb.), which had been vaccinated with a commercially prepared bacterin, were challenged by intravenous injection, and the remaining 10 were exposed by pen contact. Of the 10 unvaccinated shoats, 5 were challenged intravenously and 5 were exposed by contact (table 1). The unprotected swine were of the same age and had been housed with the vaccinated animals since the time of vaccination.

It was apparent that exposure by contact was less severe, since 4 of the 10 vaccinated shoats exposed by this method resisted the challenge completely, whereas only 1 of the 10 vaccinated pigs challenged intravenously remained normal. Of the 5 unvaccinated animals, 3 resisted exposure following contact challenge, and all of the unprotected pigs exposed intravenously showed moderate to marked febrile responses and 3 developed acute polyarthritis within the first weeks. The arthritis later (2 mo. after challenge) assumed a chronic nature in 1 animal in each of the two unvaccinated groups.

The amount of protection against contact exposure afforded by vaccination was difficult to evaluate, since the majority of unvaccinated pigs resisted the exposure, while only 1 of the 10 vaccinated pigs challenged intravenously remained normal. However, when challenged intravenously, the temperature responses were consistently slight to moderate (104.0 to 104.8 F.) in protected swine, but were marked (105.0 to 107.6 F.) in the unvaccinated animals. Since no deaths occurred in the unvaccinated pigs which were challenged by either procedure, it would appear that the swine possessed some immunity or that the culture used for challenge was of relatively low virulence. The latter proved to be true in subsequent experiments.

The incidence of acute polyarthritis (evident within 1 week after challenge) was 90 per cent in the vaccinated swine exposed intravenously, though 3 of the 9 polyarthritic animals failed to show either febrile responses or skin lesions characteristic of erysipelas. Of the 10 pigs in this group, 6 had arthritis two months postexposure. Of the 10 vaccinated pigs

TABLE 1—Results of Challenge Six Weeks Following Vaccination with Swine Erysipelas Bacterin

No. of animals	Method of exposure	Response to challenge	Condition 2 months postexposure
10 (vaccinated)	i.v.*	1 resisted exposure.	1 normal
		6, acute polyarthritis, slight to moderate fever; 4, skin lesions.	3 normal 3 arthritic
		3, acute polyarthritis.	3 arthritic
10 (vaccinated)	Contact	4 resisted exposure.	4 normal
		5, acute polyarthritis, slight to moderate fever; 2, skin lesions.	2 normal 3 arthritic
		1, acute polyarthritis.	1 arthritic
5 (unvaccinated)	i.v.	5, moderate to marked fever; 3, acute polyarthritis; 2 skin lesions.	4 normal 1 arthritic
5 (unvaccinated)	Contact	3 resisted exposure.	3 normal
		2, acute polyarthritis, moderate to marked fever; 1, skin lesion.	1 normal 1 arthritic

* The intravenous challenge consisted of 10 cc. of a 24-hour culture of *Erysipelothrix rhusiopathiae*, grown in semisolid medium.

TABLE 2—Results of Challenge 13 Weeks After Vaccination with Swine Erysipelas Bacterin

No. of animals	Method of exposure	Response to challenge	Condition 2 months postexposure
20 (vaccinated)	i.v.*	2 resisted exposure.	2 normal
		3 died (7 to 10 days).	
		12, slight to moderate fever;	5 normal
		10 had acute polyarthritis;	7 arthritic
		8, skin lesions.	
		3, acute polyarthitis.	3 arthritic
5 (unvaccinated)	i.v.	1 resisted exposure.	1 normal
		4 died (3 to 6 days).	

* Intravenous challenge.

exposed by contact, 6 developed acute polyarthritis and 1 of these developed the condition without showing any other signs of acute erysipelas. The arthritis in this animal was still evident two months after challenge. Of the 5 polyarthritic pigs which did exhibit febrile responses in this group, 3 later developed chronic arthritis.

The incidence of arthritis was much less in the unvaccinated than in the vaccinated swine two months postexposure. In the group of 5 unvaccinated pigs which were challenged intravenously, chronic arthritis developed in only 1 of the 3 animals which had acute polyarthritis. Likewise, only 1 (20%) of the 5 unvaccinated pigs exposed by contact developed chronic arthritis, as compared with 40 per cent of the vaccinated animals exposed by contact.

Thirteen-Week Challenge.—The second experiment was designed to study the effectiveness of this commercially prepared bacterin in protecting swine against challenge with *Ery. rhusiopathiae* at 13 weeks postvaccination. The degree of protection was difficult to evaluate following contact exposure at six weeks postvaccination, as a higher percentage of unvaccinated than vaccinated animals resisted contact exposure. Therefore, only the intravenous challenge was used in this trial. To assure that the culture of *Ery. rhusiopathiae* used for this challenge was in a rapidly growing state, three daily transfers were always made prior to the time of injection. The pathogenicity of the organism appeared to be increased by this method.

Of the 20 vaccinated pigs, 2 resisted the intravenous challenge (table 2), and 3 showed marked febrile responses, skin lesions typical of acute swine erysipelas, and died within ten days. Of the 5 unvaccinated animals, 4 died of the acute disease by the sixth day. The mortality was 15 per cent in vaccinated animals, as compared with 80 per cent in the unvaccinated animals, following intravenous exposure. It may be significant that, of those that died, all the vaccinated animals lived longer than did the unvaccinated animals.

Of the 15 surviving vaccinated pigs that were affected by the challenge, 3 exhibited no signs of acute erysipelas, except acute polyarthritis that later became chronic. The remaining 12 pigs showed slight to moderate febrile responses that persisted from the third to the ninth day; 10 of the 12 developed acute polyarthritis within the first week, and 8 of the 12 exhibited skin lesions of the acute disease. Two months after the intravenous challenge, 5 of the vaccinated animals that had acute polyarthritis had completely recovered, and 7 of the 12 that had febrile responses developed chronic arthritis. One of these arthritic swine died on postexposure day 70 with vegetative endocarditis.

The skin lesions in the affected swine that survived the six- and 12-week challenges were principally of the urticarial type. They were characterized by raised, well-defined edematous areas that were moderately erythematous. The characteristic diamond skin lesions were present in fewer of the surviving animals and appeared to be more evident in swine that showed slight febrile responses and were exposed by contact (6-week challenge). In no instance were skin lesions apparent in vaccinated swine that failed to show a rise in body temperature after exposure. The swine that had high temperatures (106 to 108 F.) and died following exposure had the most extensive erythema of the skin. These lesions were slightly raised, as compared with the urticarial type. They assumed a deep purple color and frequently involved more than one third of the body surface, principally the ventral side. Subnormal rectal temperatures were usual

during the terminal phase of the disease, *i.e.*, as early as 18 hours prior to death.

At necropsy, the gross pathological changes associated with acute septicemic erysipelas [17] were present. Bacteriological examinations of the spleens, livers, and kidneys of the 3 vaccinated and 4 unvaccinated pigs that died were positive for *Ery. rhusiopathiae*.

Since there was no mortality in vaccinated swine challenged intravenously at six weeks, while 15 per cent of those exposed at 13 weeks died, it would appear either that some immunity had been lost by the thirteenth week, or that the challenge at 13 weeks was more severe, possibly because of increased pathogenicity of the culture. The next experiment was designed, in part, to provide an explanation for this.

EXPERIMENT 2

In order to retest the duration of immunity produced by the bacterin used in the previous experiment, challenges were made at three months postvaccination in one group and at four months postvaccination in the second group. Housed together were 13 shoats, given subcutaneous injections of 5 cc. of the anti-swine erysipelas bacterin, and 7 control animals. In addition to the intravenous challenges, an equal number of pigs were exposed by applying *Ery. rhusiopathiae* culture to scarified skin.

Three-Month Challenge.—At the time of intravenous exposure, the 4 vaccinated swine exhibited moderate to marked anaphylactic reactions, whereas the control animal showed only a slight immediate response. The unvaccinated pig had a fever on the second day, which reached 107.4 F. on the third day. At this time, extensive erythematous areas appeared on the ventral surface of the body. The pig died on the fifth day. *Erysipelothrix rhusiopathiae* was isolated from the kidneys, liver, and spleen.

The 4 vaccinated pigs challenged intravenously developed acute polyarthritis within eight days (table 3). Of these animals, 2 showed neither skin lesions nor febrile responses, and the other 2 had mild temperature reactions (to 104.7 F.) between the second and fifth days. Skin lesions of the urticarial type were observed in only 1 of the 4 affected animals, and these lesions were slight. The acute poly-

TABLE 3—Results of Challenge Intravenously or by Scarification of the Skin Following Vaccination with Swine Erysipelas Bacterin

No. of animals	Method of exposure	Response to challenge	Condition 2 months postexposure
		THREE-MONTH CHALLENGE	
4 (vaccinated)	i.v.*	2, acute polyarthritis and slight fever; 1, skin lesion. 2, acute polyarthritis.	4 arthritic
1 (unvaccinated)	i.v.	1 died (5th day).	
2 (vaccinated)	Scarification	1 resisted exposure. 1, mild fever, skin lesion.	5, normal at the time of i.v. rechallenge
3 (unvaccinated)	Scarification	1 resisted exposure. 2, mild fever, skin lesions.	at 4 weeks, then became arthritic.
		FOUR-MONTH CHALLENGE	
3 (vaccinated)	i.v.	1 resisted exposure.	1 normal
		1, polyarthritis, slight fever, skin lesion.	1 arthritic
		1, polyarthritis.	1 arthritic
2 (unvaccinated)	i.v.	2 died (4 to 5 days).	
4 (vaccinated)	Scarification	2, skin lesions.	2 normal
		2, polyarthritis, slight fever; 1, skin lesion.	1 arthritic 1 normal
1 (unvaccinated)	Scarification	1, acute polyarthritis, slight fever, skin lesion.	1 normal

* Intravenous challenge.

arthritis became chronic in all 4 animals.

Neither the 2 vaccinated nor the 3 control animals which were challenged by scarification of the skin developed acute polyarthritis. One animal in each group resisted the exposure completely, and no differences were detected in the mild febrile responses of the other pigs. The skin reactions at the sites of inoculation were evident between the second and twelfth days and consisted, principally, of erythema and urticaria. These lesions were as pronounced and persisted as long in the vaccinated as in the unvaccinated animals.

Two weeks after the challenge by scarification, all 5 pigs were in normal health. At four weeks, when rechallenged intravenously, all exhibited marked anaphylactic reactions, but neither skin lesions nor febrile responses. It was, therefore, assumed that the 3 control swine had been protected against acute erysipelas by the topical application of *Ery. rhusiopathiae* to their scarified skin four weeks previously. However, within five days after the intravenous rechallenge, all 5 developed acute polyarthritis which, two months later, assumed a chronic nature.

By comparison of the results of intravenous challenge and exposure by skin scarification, the latter was a much milder form of challenge and was of questionable value in determining whether immunity was produced. Although the intravenous challenge was a more severe exposure than would be likely under natural conditions, it defined the protection against acute swine erysipelas afforded by vaccination.

Four-Month Challenge.—The degree of immunity produced by the bacterin apparently had not dissipated prior to the four-month challenge, since none of the vaccinated shoats died following the intravenous exposure (table 3). The 2 unvaccinated animals showed no anaphylaxis at the time of intravenous challenge, but marked febrile responses were evident on the second day (105.8 and 106.5 F.), and 1 pig died on the third and 1 on the fourth day. Both had subnormal temperatures 12 hours before death and, prior to that, had developed extensive erythematous areas in the skin, including the ears and the ventral surfaces of their bodies. At necropsy, gross pathological changes typical of acute septicemic swine erysipelas were found and *Ery. rhusiopathiae* was isolated from the kidneys, livers, and spleens of both pigs.

The 3 vaccinated shoats showed moderate to marked anaphylaxsis at the time of intravenous exposure. One animal remained in good health otherwise, while 2 developed acute polyarthritis. Of the 2, 1 failed to show either febrile responses or skin lesions. The other showed a slight rise in temperature (104.2 to 104.7 F.), which persisted until the fourth day, and mild urticarial skin lesions, which were not detectable after the sixth day. The acute polyarthritis became chronic in both hogs.

As in the three-month challenge, the exposure by scarification was so mild that it was difficult to evaluate the immunizing properties of the bacterin. Evidence of erysipelas infection appeared in all of the vaccinated as well as the unvaccinated pigs but, except for mild, local urticarial skin reactions at the sites of inoculation, 2 of the 4 vaccinated swine remained healthy. The more markedly affected animals developed a low grade polyarthritis which, after two months, became chronic in 1 vaccinated hog. The unvaccinated pig developed acute polyarthritis within a week and showed a mild rise in temperature between postexposure days 3 and 7. The arthritis disappeared by the end of the third week.

Vaccination with a commercially prepared bacterin either three or four months prior to challenge by scarification failed, in most cases, to prevent the local appearance of urticaria and hyperemia. When challenged intravenously, however, the vaccinated animals survived, while the unvaccinated animals died of acute septicemic swine erysipelas. Of the 7 vaccinated animals, 6 developed acute polyarthritis which became chronic, and 3 (50%) of the 6 developed arthritis in the absence of any other clinical signs of disease.

On subsequent intravenous challenge, all of the 5 swine that either resisted or recovered from the three-month exposure by scarification developed arthritis, and all of the vaccinated animals exhibited moderate to marked anaphylaxis, as compared with a slight or no sensitivity response in the unvaccinated hogs.

EXPERIMENT 3

The immunity produced by the subcutaneous injection of 1 cc. of desiccated

live culture of *Ery. rhusiopathiae,* concomitantly with 20 cc. of anti-swine erysipelas serum, was tested at three and four months postvaccination. In the preceding experiments, neither the challenge by contact nor by scarification provided a well-defined evaluation of the degree of protection afforded by the bacterin; consequently, only the intravenous method of challenge was used in this experiment.

Three weeks after immunization against hog cholera by the serum and virus procedure, 13 of 21 hogs housed together were vaccinated with live erysipelas culture and serum.

Three-Month Challenge.—Following the intravenous challenge, 1 of 6 vaccinated animals developed acute septicemic erysipelas and died on the eighth day (table 4). It showed a rise in temperature, to 107.8 F., and large erythematous areas in the skin. Of the remaining 5 vaccinated pigs, 2 showed slight to moderate febrile responses on the second to fourth days, and both developed acute polyarthritis, which later assumed a chronic nature. Of these 2, 1 exhibited slight urticarial lesions from the third to the eleventh days. The other 3 vaccinated pigs developed polyarthritis without any other signs of the acute disease; 1 of the 3 recovered spontaneously during the third week, but arthritis persisted in the other 2.

The protection against death afforded by the live culture and serum appeared to be marked, since 3 of the 4 unvaccinated pigs died of acute swine erysipelas in four to seven days. The vaccinated pig that died survived for eight days; however, its temperature was as high and the skin lesions were as extensive as in any of the control animals. The unvaccinated shoat that survived developed acute polyarthritis, had a moderate febrile response between the second and seventh days, and slight diamond skin lesions, *i.e.,* well-defined diamond or rhomboid-shaped erythematous areas which were not markedly raised above the surface of the adjacent skin. Within three weeks after exposure, this animal had apparently recovered.

Four-Month Challenge.—The pattern of results following this challenge was almost identical with that of the three-month challenge, and might indicate that the degree of immunity was of the same magnitude. Again, more severe anaphylactic reactions were observed in the vaccinated (sensitized) swine at the time of intravenous challenge. Of the 7 vaccinated shoats, 1 showed a marked febrile response, extensive erythematous skin lesions, and died of acute erysipelas on the eleventh day. The remaining 6 shoats developed slight to severe, acute polyarthritis in a week. Neither skin lesions nor febrile responses were observed in 3 of these 6 shoats, but chronic arthritis later developed in 2 of the 3. Of the 3 polyarthritic pigs that exhibited slight febrile responses, 2 developed slight urticarial lesions which disappeared by the ninth day, and all 3 later developed chronic arthritis.

After intravenous challenge, 3 of the 4 unvaccinated animals showed severe febrile responses from the second day to within 24 hours of death, which occurred between the third and seventh days. All 3 had subnormal temperatures (to 97.5 F.) and extensive erythematous skin lesions prior to death. As in the preceding experiment, the vaccinated shoat that died survived longer than the unvaccinated swine, although the clinical manifestations of acute swine erysipelas were similar. The visceral lesions were typical of acute septicemic erysipelas, and *Ery. rhusiopathiae* was isolated from the kidneys, livers, and spleens in every case.

The degree of protection against death was relatively good at both three and four months. The mortality in unvaccinated swine following each challenge was 75 per cent, as compared with only 15 per cent in the vaccinated animals; however, over 80 per cent of surviving vaccinated pigs developed arthritis. Although only 2 of the 8 unvaccinated control swine survived the intravenous challenge and both had acute polyarthritis, neither developed chronic arthritis.

DISCUSSION

The amount of protection afforded to swine by a commercially prepared erysipelas bacterin of the "Traub" type was difficult to evaluate in swine challenged intravenously six weeks after vaccination, since only 1 of 10 animals resisted the exposure. Although several of the unvaccinated swine showed slightly more pronounced febrile responses, the differences were not marked. The incidence of skin lesions was the same in both groups, and none of the unvaccinated swine died. This suggested that either they possessed some

TABLE 4—Results of Challenge After Vaccination with Desiccated Live Culture and Anti-Swine Erysipelas Serum

No. of animals	Method of exposure	Response to challenge	Condition 2 months postexposure
		THREE-MONTH CHALLENGE	
6 (vaccinated)	i.v.*	1 died (eighth day).	2 arthritic
		2, acute polyarthritis, slight to moderate fever; 1, skin lesion.	2 arthritic
		3, acute polyarthritis.	1 normal
4 (unvaccinated)	i.v.	3 died (4 to 7 days).	1 normal
		1, acute polyarthritis, moderate fever, skin lesion.	
		FOUR-MONTH CHALLENGE	
7 (vaccinated)	i.v.	1 died (eleventh day).	
		3, acute polyarthritis, slight fever; 2, skin lesions.	3 arthritic
		3, acute polyarthritis.	2 arthritic
			1 normal
4 (unvaccinated)	i.v.	3 died (3 to 7 days).	
		1, acute polyarthritis, marked fever.	1 normal

* Intravenous challenge.

natural immunity or the challenge was made with cultures of relatively low virulence.

Mortality can not always be used as a criterion for evaluating susceptibility or immunity to erysipelas infection, since mortality frequently may be low following natural infection. Nevertheless, at the 13-week intravenous challenge, a well-defined protection was evident; 80 per cent of the unvaccinated pigs died of acute septicemic erysipelas, as compared with 15 per cent of the vaccinated animals. On the basis of these two trials, it appeared that some protection may have been lost in 13 weeks, because there was no mortality in the group challenged at six weeks. These findings would seem consistent with the results of Sikes *et al.*, [19] who reported that the protection afforded by bacterins of this type had waned prior to 12 weeks.

In the second experiment (table 3), swine were vaccinated with the same bacterin used in the first experiment and challenges were made, intravenously and by scarification of the skin, at three and four months postvaccination. Since no loss in immunity was apparent prior to the latter challenge, the authors believe that changes in the virulence of the culture of *Ery. rhusiopathiae* may account for the discrepancy.

In all of these experiments, neither the contact challenge nor the exposure by scarification of the skin proved to be of appreciable value in determining the effectiveness of immunity. In the swine exposed by contact, in the first experiment, a higher percentage of unvaccinated than of vaccinated animals resisted the exposure, though the febrile responses in the affected unvaccinated pigs were somewhat more pronounced. In those exposed by scarification, an unvaccinated animal occasionally exhibited somewhat higher temperature than did the vaccinated pigs, but none died. The skin lesions were generally mild, principally of the urticarial type and, in all cases, were most apparent in the areas of scarification. Vaccination failed to prevent these local reactions. There was no difference between the lesions in vaccinated and unvaccinated swine.

When intravenous challenge was used, the susceptibility of the unvaccinated swine was almost 100 per cent, and 14 of 21 (66%) died. Although intravenous challenge is more severe than a natural exposure, this procedure was believed to be the most suitable, within the limits of these experiments, for evaluating the degree of protection afforded by vaccination.

It seems significant that, as in a previous study, a high percentage of vaccinated swine developed acute polyarthritis which, in most cases, became chronic. Of the 50 vaccinated swine in the various groups which were exposed intravenously, 31 (62%) had arthritis two months after challenge, whereas only 1 (14%) of the 7 surviving unvaccinated animals challenged in this way developed chronic ar-

thritis. It is difficult to make valid comparisons from these data, because only 7 of the 21 unvaccinated swine survived intravenous exposure.

In previous experiments [9,17] in which 191 animals were used, we were able to produce swine erysipelas in susceptible swine by seven different exposure procedures, including exposure by contact, scarification of the skin, intradermal, intravenous, intra-articular, intramuscular, and subcutaneous injections. Approximately one third of the pigs died of the acute disease, one third either resisted the exposure or recovered completely, and one third developed chronic arthritis.

The 62 per cent incidence of chronic arthritis in the vaccinated (sensitized) swine lends support to the belief that sensitization may be an etiological factor in chronic joint disease following erysipelas infection. Furthermore, vaccinated pigs consistently exhibited more anaphylaxis at the time of intravenous challenge. Anaphylactic reactions are similar to allergic or hypersensitivity reactions, and observations suggest that the two are fundamentally allied.[4]

The culture medium, in which the organism was grown and which was subsequently used for challenge, contained 10 per cent horse serum. The immunized swine in experiment 3 (table 4) received anti-swine erysipelas serum of equine origin simultaneously with desiccated live cultures of *Ery. rhusiopathiae*. Could the anaphylaxis that occurred at the time of intravenous exposure reflect a hypersensitivity to horse serum, or were the allergins of *Ery. rhusiopathiae* responsible for the anaphylactic reactions?

In current experiments, the authors were able to produce marked allergic reactions in swine by weekly intravenous injections of 1 cc. of sterile horse serum. Anaphylactic responses were evident at the time of the fourth weekly injection (21 days) and, subsequently, at the time of each weekly intravenous injection. After a series of ten injections, the swine were allowed to go untreated for four weeks and, when rechallenged on the fifth week, no shock reactions were evident. It was concluded that, although swine could be hypersensitized to horse serum, specific antibodies produced in this manner rapidly decreased, to the extent that anaphylaxis failed to occur after 28 days.[10] The pattern of hypersensitivity was essentially the same in swine sensitized by weekly injections of heat-killed cultures of *Ery. rhusiopathiae*. However, in this case, swine showed marked anaphylactic reactions when rechallenged eight weeks after the last in a series of ten weekly intravenous injections.[10] It was evident that the allergins of this organism were capable of producing a more lasting sensitivity.

It was assumed that the anaphylactic responses that occurred in vaccinated swine at the time of intravenous challenge were a manifestation of hypersensitivity to the allergins of *Ery. rhusiopathiae*. In experiment 3, the time between the sensitizing dose (vaccination) and the provocative dose (challenge) was three and four months.

Further evidence that sensitivity is involved in the mechanism by which *Ery. rhusiopathiae* causes chronic proliferative arthritis in swine is suggested by the fact that numerous investigators have found it necessary to use repeated injections of the organism.[1,7,22,23] Recently, Hughes [7] was able to produce the disease in all of 16 pigs, without apparent signs of acute swine erysipelas, by repeated intravenous injections. Only 1 or 2 pigs showed temperature responses of 104.0 to 104.4 F.

Similarly, Usdin *et al.*[22] produced arthritis in 7 of 8 swine without overt signs of acute septicemic erysipelas, *i.e.*, marked febrile responses and skin lesions. Sikes *et al.*[20] produced chronic arthritis in all of 4 swine, which resisted an initial intravenous exposure, by giving repeated weekly intravenous injections of *Ery. rhusiopathiae*. Of the 4, 3 developed vegetative endocarditis, which apparently caused the deaths of 2 of them. In the previous reports,[7,22] the pathological changes in the joints were produced in the absence of febrile responses and skin lesions. Likewise, in the present study, acute polyarthritis and ultimate chronic joint disease frequently occurred without other manifestations of acute swine erysipelas.

Recently, Goldie and Collins [5] were successful in producing arthritis in rabbits, which was pathologically similar to the disease in swine, by giving repeated intravenous injections of *Ery. rhusiopathiae*. These authors emphasized that, in most instances, the exposures were too small (7 to 28 million organisms) to produce other pathological effects. Hypersensitivity induced in rabbits by massive injections of

horse serum occasionally resulted in arthritides in conjunction with cardiovascular lesions.[8] Pearson[12,13] recently reported the development of arthritis, periarthritis, and periostitis in rats that received adjuvants of allergenic emulsions. It was believed that hypersensitivity was probably the basis for these reactions; however, the possibility of pleuropneumonia-like organisms had not been excluded.

According to Usdin *et al.*,[22] arthritis is common in horses which are hyperimmunized with living cultures of *Ery. rhusiopathiae* for the commercial production of anti-swine erysipelas serum.

The production of arthritis in swine by repeated exposure to *Ery. rhusiopathiae* and, in the present study, by vaccination and subsequent challenge, where overt signs of acute erysipelas are absent, would seem analagous to the arthritis which arises naturally in pigs which have been visibly ill from the acute disease. In view of the wide distribution of the swine erysipelas organism in species other than swine[2,24] and in swine that are apparently healthy,[3,6] it seems probable, under farm conditions, that some pigs develop arthritis, without other manifestations of infection, through sensitization by multiple natural exposures.

Whether joint disease results from an attack of acute swine erysipelas or develops without signs of disease, it is reasonable to assume, according to Hughes,[7] that arthritis usually arises as a result of a bacteremia. Although *Ery. rhusiopathiae* reaches the joints via the peripheral circulation, we are not certain that the pathological changes are a direct result of the presence of the organism. Hughes further suggests that, if arthritis results from a single exposure, the joints may receive repeated doses from some other foci in the body, which are set up by the exposure.

In the experiments of Sikes *et al.*[17,18] and Neher *et al.*,[9] arthritis was produced following a single exposure; however, the pathological changes in the joints were, in this instance, a sequel to acute swine erysipelas, in which roughly 90 per cent of the animals were acutely ill. In general, the mortality rate approximated 33 per cent and, of the surviving animals, approximately 50 per cent developed chronic arthritis.

The sensitivity theory may explain, in part, why some affected joints are apparently sterile.[1,7,9,17,18,22] We were able to isolate *Ery. rhusiopathiae* from more than 70 per cent of the affected joints, examined from experimental cases in which infection was established less than five months previously; however, it was increasingly difficult after this time, though the pathological process usually continued unabated. In no instance were we able to isolate the organism after 226 days following experimental exposure.[17]

In man, numerous clinical observations support the view that allergic manifestations are closely related to some of the so-called rheumatic or collagen diseases[21] and to the hyalinoses that are regarded as diseases of adaptation.[16] This group includes rheumatic fever, rheumatoid arthritis, periarteritis nodosa, lupus erythematosus disseminatus, scleroderma, dermatomyositis, and erythema nodosum.[15] There is more evidence to support sensitivity as an etiological factor in erythema nodosum and periarteritis nodosa than in the other members of the group.

SUMMARY AND CONCLUSIONS

Of the 96 swine used in these experiments, 66 were vaccinated against swine erysipelas (53 with aluminum hydroxide, adsorbed type, 13 with desiccated live culture and serum), and 30 were used as unvaccinated controls. Challenges were made at various times from six weeks to four months after vaccination.

Intravenous challenge was superior to exposures by contact and scarification of the skin as a method of demonstrating the degree of protection afforded.

Although vaccination did not prevent the establishment of erysipelas infection following challenge, it did prevent the high mortality that occurred in the controls. Essentially, there was no difference in effectiveness; some protection was evident for at least four months following vaccination with either product.

It was concluded that vaccination will not prevent arthritis, since a higher incidence consistently resulted in vaccinated pigs. Of the 50 protected animals which were exposed intravenously, 31 (62%) had rheumatoid arthritis two months after challenge, and only 1 (14%) of 7 surviving unvaccinated pigs developed chronic arthritis. Sensitization may be an important etiological factor in arthritis, particularly since marked anaphylactic reactions were evident in vaccinated swine at the

time of injection, in contrast to negative or slight allergic responses in the controls.

The role of hypersensitivity and the production of arthritis without other manifestations of acute erysipelas, as reported here, are discussed in conjunction with naturally occurring arthritis in swine which have never been visibly ill with acute septicemic erysipelas.

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The Role of Hormones in the Relaxation of the Uterine Portion of the Cervix in Swine

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THIS PAPER deals with one phase of a study designed to establish the possibility of using hormones to induce labor in sows. If parturition could be made to occur at the convenience of the herdsman, during daylight hours, the method might have considerable practical appeal. It seems most important to establish the effect of hormones on the uterine, especially the cervical, musculature and on the ability of the organs associated with parturition to relax when they are stimulated by the proper hormones.

While the literature on the effect of hormones on the relaxation of the symphysis pubis (especially in laboratory animals) is voluminous, relatively little is known about the role of hormones on the relaxation of the uterine portion of the cervix. An investigation on this subject is reported here.

The corpus luteum of the ovary and the uterine endometrium are known to produce a protein hormone, relaxin, which causes relaxation of the symphysis pubis in many laboratory animals.[2] Graham and Dracy[3, 4] have found that relaxin injected into cows previously treated with estrogen causes a minimum relaxation of the cervix when 250 guinea pig units (GPU) of relaxin are injected and a maximum relaxation after the injection of 1,500 GPU. Zarrow, Sikes, and Neher,[5] using 2 animals, showed that stilbestrol (5 mg. daily for 10 days) causes partial relaxation of the cervix of sows while, if the estrogen is followed by 500 GPU of relaxin (3 times daily for 4 days), a complete relaxation of the cervix can be obtained.

MATERIALS AND METHODS

To determine the degree of relaxation of the uterine portion of the cervix, a series of 16 aluminum rods 12 inches in length was used. Their diameters varied by ⅛ of an inch, from ⅛ to 2 inches. One end of each rod was tapered at a 45-degree angle. To obtain the measurements, a sow was confined in a crate, a rod was lubricated with mineral oil and inserted carefully through the vagina into the cervix. If the end entered without resistance, a rod of the next larger size was inserted. After a little practice, it became relatively easy to estimate the maximal size of the rod which would slip into the cervix easily yet tightly. Care was taken never to exert force with a rod. Most experimental sows did not object to measurements being made once and, occasionally, twice a day and there was no indication that the frequent taking of measurements altered length of cycles or interfered with ovulations.

The accuracy of measurements was verified in many instances, in different stages of the cycle, by inserting the rods into the cervix and marking the protruding end of the rod at its deepest penetration, then slaughtering the animal and reinserting the rod into the excised reproductive tract.

The experiments were done in three stages, using 16 crossbred sows and gilts. In the first stage, the females were checked for heat daily with a vasectomized boar and were allowed to go through a normal cycle without being measured. They were then measured daily through one or more estrous cycles to establish the normal variations in cervical relaxation.

Some of the sows were oophorectomized and measurements on cervical size were continued for varying times after the operation. In the final phase of the experiment, castrated sows were injected with the different hormones (estrogen, progesterone, and relaxin) in various sequences and combinations and with varying dosages. The hormones used were: theelin, estradiol 17-cyclopentylpropionate (ECP), progesterone, and relaxin.* The relaxin was standardized in guinea pig units, 1 unit being the minimum quantity required to relax the pubic symphysis of two-thirds of 20 guinea pigs injected.

RESULTS

Normal Cycle.—Measurements made during the normal cycle (graph 1, composite graph of measurement made on 9 sows) were contrary to expectation. It is generally stated that the cervix of the sow relaxes completely during estrus and that the penis of the boar actually penetrates it. Ejaculation is reported to occur into the cervix[1] and possibly even into the uterus. Our results indicate that the cervix not only does not relax during estrus but is most constricted at that time. The

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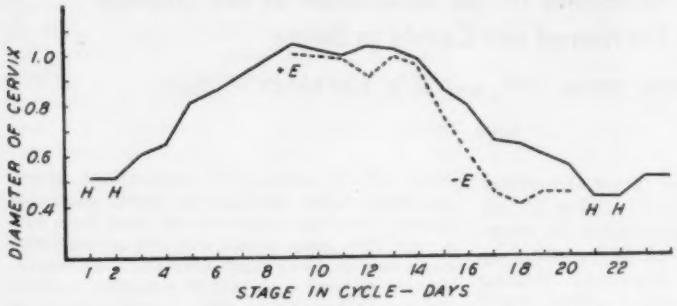
* Releasin was prepared and generously supplied by the Warner-Chilcott Research Laboratories, Morris Plains, N. J.



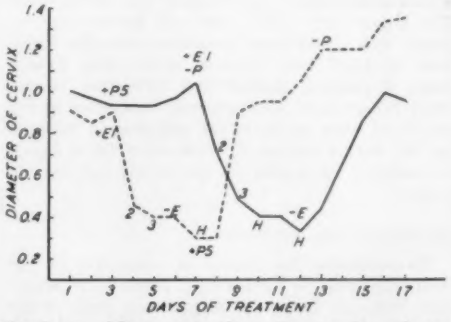
Graph 1—Degrees of relaxation of vaginal part of cervix during the normal estrous cycle of sows. The greatest constriction occurs during estrus. Exogenous estrogen can hasten constriction of the cervix by a few days (dotted line). H indicates days of heat; +E indicates onset of estrogen injection, and −E indicates end of estrogen.

greatest relaxation occurs on day 9 of the cycle and is maintained until day 13 when the cervix begins to constrict again (graph 1). This pattern was uniform in 9 females and led to the assumption that estrogen is responsible for cervical constriction, while progesterone causes its relaxation.

To test the ability of estrogen to cause cervical construction, injections of ECP were begun on day 9 of the normal cycle, well before constriction would be expected to begin. Five intact sows were injected with 1 mg. of ECP on day 9, 2 mg. on day 10, and 3 mg. on day 11, the latter dose being repeated daily until day 16. While there was no immediate response to this treatment, it appears that maximal cervical constriction was reached a little more rapidly and about two days earlier than it would have in untreated sows (graph 1, compare solid and dotted curves).

Castration and Hormone Therapy.—Castration caused a rapid, complete, and sustained relaxation of the cervix. There was no evidence of variation in the degree of cervical dilatation and, when ECP was injected, complete cervical constriction followed; progesterone had no measurable effect. Withdrawal of ECP resulted in rapid cervical dilatation (graph 2). Large doses of progesterone (up to 20 mg. daily) were not able to overcome the constricting effect of estrogen and 750 GPU per day of relaxin did not cause cervical relaxation when given concurrently with estrogen (graph 3). It is possible that the dose of relaxin employed was not sufficiently high to indicate its ability to relax a cervix constricted by estrogen. Further experiments with larger doses of relaxin, administered more frequently, are needed. That progesterone is unable to cause cervical constriction is indicated (graph 3).

Anatomy of the Cervix and Site of Semen Deposition.—In the course of these experiments, particularly those in which the degree of cervical relaxation was checked after slaughter, the porcine cervix was found to consist of two distinct anatomical portions, the uterine portion, which is 4 to 5 cm. long, and the vaginal portion, which is approximately 10 to 12 cm. in length. Both portions have annular rings and are histologically similar. The major difference lies in the fact that the shorter, uterine, portion apparently remains tightly closed at all times and does not relax even during the mid-cycle. The vaginal portion undergoes cyclic closure and relaxation. The tightly closed uterine portion was probably never penetrated even by our



Graph 2—After castration, the cervix relaxes completely and remains relaxed at all times. Solid line shows that progesterone alone (5 mg./day) is ineffective and that the cervix remains constricted as long as estrogen injections continue. The dotted line again demonstrates the effectiveness of estrogen (E) and the inability of progesterone (P) to prevent cervical relaxation after estrogen withdrawal. H indicates heat. +E to −E indicates duration of daily estrogen injections and +P to −P, the duration of progesterone injections. Numbers 1, 2, 3, or 5 indicate the daily dose in milligrams of the hormones used.

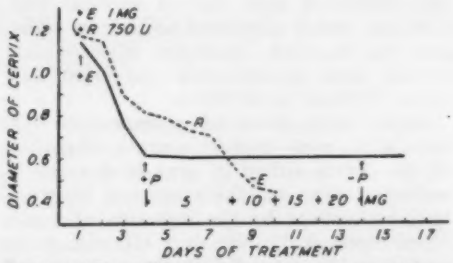
smallest probes because even in excised reproductive organs it was necessary to hold the cervix with one hand and "thread" the probe through with the other.

Experiments were also performed to determine the probable site of semen deposition in pigs. The whole length of the cervix of a gilt was injected with radiopaque material in such a manner as to deposit a 20 per cent solution of thorium-oxide under the serosa of the cervix. Thorium-oxide was also injected into the glans penis of a boar. Radiographs were taken from the flank when the animals mated and throughout copulation. The relative positions of the thorium-oxide blebs in the penis and the cervix clearly showed that the penis penetrated into the anterior part of the vaginal portion of the cervix, but not into the tightly closed uterine portion, and that ejaculation occurred into about the middle of the vaginal portion and not into the uterus.*

DISCUSSION

The results presented here are in contradiction to those obtained by other workers on pigs [5] and cattle.[3,4] Our data, based on a large number of animals and of measurements, indicate that relaxation of the cervix during mid-cycle and its constriction during estrus are constant phenomena. The data also show that constriction of the cervix is due to estrogen, while relaxation is not due to the presence of a hormone but is the result of the absence of estrogen. Further work is needed to determine whether the failure to induce cervical dilatation with relaxin was due to insufficient doses or failure to build up a high blood level of relaxin by frequent injections.

The fact that estrogen causes cervical constriction while its absence causes relaxation raises the question whether the greater susceptibility of the uterus to infection frequently ascribed to the presence of progesterone may not be due, in part, to the fact that in the absence of estrogen part of the cervix is wide open, facilitating bacterial invasion of the uterus. Conversely, greater uterine resistance to infection during estrogen stimulation may



Graph 3—The solid line shows that even as much as 20 mg. of progesterone daily did not counteract the effect of estrogen (1 mg. of ECP, injected daily from day 1 through 16) on maximal cervical constriction. The dotted line shows that 750 units of relaxin daily did not counteract the effect of 1 mg. of ECP daily. This dose of relaxin may decrease the rate at which the cervix constricts in response to ECP. +E to −E indicates duration of estrogen injection; +R to −R indicates duration of relaxin injection; and +P to −P indicates duration of progesterone injection.

be the result of tighter cervical closure during that time.

Since the cervix is tightly constricted at the time of estrus, it appears improbable that ejaculation occurs directly into the uterus. Radiographs reveal that the glans penis penetrates to a depth of a few centimeters, or about midway, into the vaginal part of the cervix. The anatomical peculiarities of the penis in relation to the annular rings of the vaginal cervix, and actual observations made during mating, make it appear probable that the penis is actually gripped by the annular rings and that ejaculation does not occur until such a union has occurred.

SUMMARY

Anatomically, the uterine cervix in swine was found to consist of two portions, the long, vaginal portion, which constricted and relaxed cyclically, and the short, uterine portion which remained tightly closed.

Daily measurements of the diameters of the cervices of 9 normal sows showed that the vaginal portion was constricted during days 1 and 2 of estrus and then relaxed gradually until about day 9 of the estrous cycle with gradual constriction beginning again about day 14.

After oophorectomy, there was complete relaxation of the vaginal portion of the cervix, while the uterine portion was not noticeably affected. Injection of estrogen

* We are indebted to Drs. T. N. Phillips and P. J. Dziuk for performing this experiment and making the information available to us.

into castrated sows caused cervical constriction which continued as long as estrogen was injected. Estrogen injected into normal sows in mid-cycle somewhat hastened cervical constriction.

Even large doses of progesterone (20 mg. daily) were unable to cause relaxation of the cervix either in sows in heat or in castrated sows in which cervical constriction was caused by the injection of 1 mg. of estrogen daily. Relaxin (Releasin) injected at the rate of 750 GPU per day did not cause cervical relaxation. Experiments on the effect of relaxin on the cervix are being continued.

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The Use of Piperazines as Anthelmintics for Swine

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PIPERAZINES have been used with increasing frequency for the control of helminthic infections in swine since the demonstration of their anthelmintic action in these animals by Leiper [7] and Sloan *et al.*[11] in 1954. These compounds have a wide margin of safety for the host; they are active against nodular worms, as well as against mature and immature ascarids; and they apparently present no hazard to man, either in themselves or as residual deposits in edible tissues. Nevertheless, as stated previously,[1] a major limiting factor in their practical application as general anthelmintics for these animals is the problem of assuring adequate dosing under field conditions.

The piperazines are conveniently given in feed or drinking water; but dosages are calculated on the basis of body weight and, presumably, must be consumed within a comparatively short period in order to achieve maximum efficiency at minimum cost. To attain this objective, the animals are usually deprived of either feed or water (whichever is to be used as the vehicle) 12 to 18 hours before treatment, and the calculated dose is mixed in an amount of feed, or water, which is somewhat less than that normally consumed in one day. It is possible, therefore, that the more vigorous and probably less heavily parasitized animals may obtain a disproportionate share of highly palatable medicated feed or water. Conversely, less palatable mixtures may be consumed reluctantly and result also in inadequate dosing of some animals. The unreliability of medication by means of drinking water is further attested by the marked variations in water consumption occasioned by seasonal or daily differences in atmospheric temperature, or for other reasons. These factors obviously have a direct influence on the concentration and, probably, palatability of drugs that are given in the drinking water.

It may be particularly significant that published reports [1, 3, 4, 6-10, 12, 13] of critical trials establishing the marked efficiency of currently recommended doses of piperazine against ascarids and nodular worms in swine have usually involved only individually treated animals, whether the chemicals were given in feed or drinking water. Moreover, the widely accepted optimum dosage of piperazine for swine, *i.e.*, 50 mg. per pound of body weight, appears to have been established on a similar basis. This method of determining efficiency and optimum dosage seems neither rational nor technically sound. The possible hazard of attempting definitive applications of information obtained in this manner is suggested by the findings of Leiper,[7] whose limited trials with piperazine-1-carbodithioic acid showed that efficacy against both ascarids and nodular worms was lower in group trials than in animals treated individually. Similar findings with respect to large roundworms were reported by Wertejuk and Chowaniec [12, 13] in 1956, but the efficiency against nodular worms was apparently low in all trials. Thus, it is evident that definitive applications of chemotherapeutic agents and measures must be proved by acceptable testing procedures.

It seemed desirable, therefore, to obtain critical data on the anthelmintic action of several piperazine compounds administered in feed and drinking water to groups of pigs, to compare the performance with that attained by individual dosing, and to assay the efficiency of different preparations under comparable conditions and in equivalent piperazine dosages. (Some of the data were summarized in an earlier report.[2])

MATERIALS AND METHODS

The pigs used were of mixed breeds and were 2 to 6 months old. Most of them harbored naturally acquired gastrointestinal helminths, commonly found in swine in this area, although experimental infections of the large roundworm, *Ascaris lumbricoides*, and nodular worms, *Oesophagostomum* spp., were established in some instances. In all cases, the pigs were confined at least two days before treatment, individually or in groups, in concrete-floored pens, and the feces were screened daily for naturally eliminated parasites. This also permitted specific determination

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of feed and water consumption for each animal or test group.

In the several trials, the chemicals were given in the feed or drinking water, in accordance with commonly accepted practices and recommendations. The feces were collected at frequent intervals each day and examined for parasites in the usual manner. When the elimination of parasites ceased, usually within four to seven days after treatment, the pigs were slaughtered and the entire gastrointestinal tract examined for parasites and lesions.

EXPERIMENTAL PROCEDURE AND RESULTS

*Piperazine Adipate.**—The results show (table 1) that doses providing 50.4 and 67.3 mg. of piperazine per pound of body weight removed all of 139 ascarids *(A. lumbricoides)* from 14 pigs, and 99 per cent of 2,868 nodular worms (Oesophagostomum spp.) from 8 pigs, when treated individually. The efficacy against both species was substantially lower, however, in animals that were treated in groups. In a group of 4 pigs that was given the smaller dosage without preparatory fasting, the chemical removed only 76 per cent of 94 ascarids and 69 per cent of 6,202 nodular worms. Moreover, only 1 of the 4 pigs was free of large roundworms at necropsy. A similar dosage, given to 4 pigs after a preliminary fast of 18 hours, removed 81 per cent of 63 ascarids and 72 per cent of 6,056 nodular worms (not a significant increase) and 3 of the animals were infected with ascarids at the end of the test period. Two additional groups of 4 pigs each were given the chemical at the slightly higher dosage of 55.5 mg. of piperazine per pound of body weight. When given to one group in the feed, at least 21 per cent of 117 ascarids and 26 per cent of 922 nodular worms were removed. When given to the other group in the drinking water, at least 54 per cent of 22 ascarids and 73 per cent of 4,343 nodular worms were removed. The efficacy against ascarids in both trials is undoubtedly conservative, because some of the worms were eaten by the pigs before they could be collected. Nevertheless, at necropsy, the 3 pigs in the first group harbored a total of 92 and 1 pig in the second group harbored 10 large roundworms.

There was no demonstrable action against red stomach worms *(Hyostrongylus rubidus)*, thick stomach worms *(Ascarops strongylina)*, or whipworms *(Trichuris suis)* in any of these trials. No attempt was made to recover Hyostrongylus in the feces after treatment, but the pigs harbored an average of 234 each at necropsy. The chemical was ineffective against a sin-

* The piperazine adipate was supplied by Carson Chemicals, Inc., New Castle, Ind., and by William Cooper and Nephews, Chicago, Ill.

TABLE 1—Data on Piperazine Adipate Given in Feed or Drinking Water

Piperazine dosage (mg./lb.)	No. of pigs	Parasites	Removed	Left	Efficacy (%)
67.3	4(a)(b)	Ascaris	80	0	100
		Trichuris	5	241	2
	2(a)	Ascaris	7	0	100
		Trichuris	2	239	1
50.4	8(a)	Ascaris	52	0	100
		Oesophagostomum	2,849	19	99
		Hyostrongylus	....	1,526	....
		Trichuris	4	147	3
	4	Ascaris	72	22(3)‡	76
		Oesophagostomum	4,288	1,914	69
		Hyostrongylus	....	2,048	....
		Trichuris	1	4	20
	4(c)	Ascaris	51	12(3)	81
		Oesophagostomum	4,340	1,716	72
		Hyostrongylus	....	2,938	....
		Ascarops	0	1	0
55.5†	4	Ascaris	25*	92(3)	21*
		Oesophagostomum	241	681	26
		Hyostrongylus	....	178	....
		Trichuris	12	28	30
	4(d)	Ascaris	12*	10(1)	54*
		Oesophagostomum	3,165	1,178	73
		Hyostrongylus	....	330	....
		Trichuris	19	566	3

* Additional worms eaten by pigs, percentage efficacy conservative; † recommended dosage of commercial concern; (a) treated individually; (b) published data, see reference 1; (c) fasted 18 hours; (d) medicated drinking water, treated 4-23-56; ‡ number of pigs infected at necropsy.

gle Ascarops and removed only 3 per cent of 1,268 whipworms in the several trials.

*Piperazine-1-Carbodithioic Acid.**—The data on anthelmintic action (table 2) show that, in limited individual trials with a dry feed mix, therapeutic doses of the chemical removed 72 per cent of 67 ascarids from 3 pigs and 99 per cent of 3,998 nodular worms from 4 pigs; none of the 3 infected pigs were free of ascarids at necropsy. In the several trials with groups of 2 to 4 pigs, 64 per cent of the 443 ascarids and 84 per cent of 6,425 nodular worms were removed. Complete removal of large roundworms was obtained in the 8 pigs treated in pairs, although only a single ascarid was recovered in two instances. Moreover, no ascarid eggs were found in the feces of these 4 pigs before treatment, and none were found in the feces of 1 of the pigs in a third pair. Significantly, perhaps, a total of 157 large roundworms were recovered at necropsy from 5 of the 10 pigs that were treated in groups of 3, 3, and 4 animals, respectively.

In individual and group trials with therapeutic doses of a solution of the chem-

* The piperazine-1-carbodithioic acid (Parvex) products were supplied by the Upjohn Company, Kalamazoo, Mich.

TABLE 2—Data on Piperazine-1-Carbodithioic Acid Given in Feed or Drinking Water at 125 mg. per Kilogram of Body Weight

No. of pigs	Parasites	Removed	Left	Efficacy (%)
4(a)(b)	Ascaris	48	19(3)‡	72
	Oesophagostomum	3,964	34	99
	Hyostrongylus	....	1,349	....
	Ascarops	0	1	0
	Trichuris	1	4	20
2	Ascaris	39	0	100
	Oesophagostomum	996	16	98
	Hyostrongylus	....	327	....
2	Ascaris	1	0	100
	Oesophagostomum	596	198	75
	Hyostrongylus	....	714	....
	Trichuris	0	9	0
2	Ascaris	32	0	100
	Oesophagostomum	885	328	73
	Hyostrongylus	....	369	....
	Trichuris	0	39	0
2	Ascaris	1	0	100
	Oesophagostomum	1,022	184	85
	Hyostrongylus	....	78	....
	Trichuris	0	26	0
3	Ascaris	47	91(1)	34
	Oesophagostomum	16	17	48
	Trichuris	2	202	1
3	Ascaris	25	58(2)	30
	Ascarops	0	25	0
	Trichuris	3	169	2
4	Ascaris	141	8(2)	94
	Oesophagostomum	1,886	281	87
	Hyostrongylus	....	572	....
	Trichuris	27	98	22
1(c)	Ascaris	0	2	0
	Oesophagostomum	260	223	54
1(c)	Ascaris	8	1	88
	Oesophagostomum	740	236	76
	Hyostrongylus	....	98	....
4(c)	Ascaris	125	5(2)	96
	Oesophagostomum	1,987	1,862	51
	Hyostrongylus	....	513	....
	Trichuris	7	187	4
2(d)	Ascaris	2	0	100
	Trichuris	1	0	100
3(d)	Ascaris	3	35(2)	8
	Trichuris	0	2	0

(a) Treated individually; (b) 1 pig not infected with ascarids when treated; (c) solution in drinking water, treated March, 1956; (d) suspension in drinking water, treated March, 1957; ‡ number of pigs infected at necropsy.

ical given in drinking water, 94 per cent of 141 ascarids and 56 per cent of 5,308 nodular worms were removed from 6 pigs. The medicated water was extremely distasteful. Before treatment, the group of 4 pigs consumed about 12.5 qt. of water daily. After withholding water for 18 hours, the recommended dosage of the chemical was given in about 7 qt., a little more than half the normal daily consumption. Only 4 qt. of the medicated water was consumed during the first 24 hours, and about a pint remained after 48 hours. On several occasions, the pigs were observed drinking urine and when fresh water was given, after the 48-hour period, they consumed approximately 14 qt. within four hours.

An aqueous suspension of the chemical was given in the drinking water to two groups of 2 and 3 pigs, respectively. The treatment was completely effective against 2 ascarids in the group of 2 pigs but only 8 per cent of 38 ascarids were removed from the group of 3 pigs. However, 37 of 38 ascarids in the latter trial were immature, ranging from 1 to 3 cm. in length. The only mature ascarid in the 3 animals was recovered at necropsy. In these trials, also, the medicated water was consumed reluctantly; and the pigs drank nearly twice the normal amount of fresh water during the first 24 hours after treatment.

There was no demonstrable action against red stomach worms, thick stomach worms, or whipworms in any of these trials. The chemical was wholly ineffective against 26 Ascarops and only 5 per cent of 774 whipworms were removed in the several trials. In most cases, no attempt was made to recover Hyostrongylus in the feces after treatment, but an average of 122 per pig was recovered at necropsy.

Comparative Trials.—In all trials (table 3), the animals were deprived of water for about 18 hours before treatment. Except in tests with the commercial mixture of piperazine hydrochlorides, the appropriate dose was given in approximately three fourths of the amount of water usually consumed daily by each group. With

TABLE 3—Data on Various Piperazine Salts Given in Drinking Water at the Rate of Approximately 50 mg. of Piperazine Base per Pound of Body Weight

Piperazine salt	Date of treatment	No. of pigs	Parasites	Removed	Left	Efficacy (%)	Water consumption (ml.) Before treatment	When containing drug	% drug consumed/ No. hrs.
Citrate	9/10/56	4	Ascaris	23*	0	100	11,000	8,250	100/24
			Oesophagostomum	935	20	98			
			Hyostrongylus	....	94	....			
			Ascarops	0	7	0			
			Trichuris	9	305	3			
Dihydrochloride	9/17/56	4	Ascaris	223	35(1)‡	86	5,600	4,200	100/24
			Oesophagostomum	6,386	50	99			
			Hyostrongylus	....	220	....			
			Ascarops	0	37	0			
			Trichuris	38	460	8			
Sulfate	9/24/56	4	Ascaris	66	9(2)	88	6,500	4,875	85/24
			Oesophagostomum	590	390	60			
			Hyostrongylus	....	114	....			
			Ascarops	0	1	0			
			Trichuris	17	222	7			
	3/11/57	2	Ascaris	11	0	100	4,150	3,100	100/7
			Oesophagostomum	1	0	100			
Adipate	10/1/56	4	Ascaris	141	63(4)	69	4,400	3,300	81/24
			Oesophagostomum	233	106	69			
			Hyostrongylus	....	160	....			
			Trichuris	4	212	2			
Mono- and dihydrochloride mixture†	4/2/56	4	Ascaris	17	30(1)	36	11,000	6,000	33/24
			Oesophagostomum	724	1,263	36			35/48
			Hyostrongylus	....	479	....			
			Trichuris	86	97	47			
	4/16/56	4	Ascaris	15	48(1)	24	9,600	9,600	42/6
			Oesophagostomum	1,597	1,308	55			64/24
			Hyostrongylus	....	458	....			100/48
			Trichuris	0	14	0			

* Additional worms eaten by pigs; † commercial product; ‡ number of pigs infected at necropsy.

piperazine citrate,* all of at least 23 ascarids and 98 per cent of 955 nodular worms were removed from 4 pigs; additional ascarids were eaten by the pigs before they could be collected. The pigs consumed all of the medicated water within 24 hours. Using piperazine dihydrochloride,† 99 per cent of 6,436 nodular worms but only 86 per cent of 258 ascarids were removed from 4 pigs, although the group consumed the entire amount of medicated water within the 24-hour period of treatment. All of the 35 ascarids that were unaffected by the treatment were found in 1 pig. With piperazine sulfate,‡ 88 per cent of 75 ascarids and 60 per cent of 980 nodular worms were removed from one group of 4 pigs. These animals consumed only 85 per cent of the medicated water during the treatment period, and 8 of the 9 ascarids and 387 of the 390 nodular worms recovered at necropsy were in 1 animal. In a subsequent trial, all of 11 ascarids and 1 nodular worm were removed from a group of 2 pigs that consumed all of their medicated water within a period of only seven hours. With piperazine adipate, 69 per cent of 204 ascarids and 69 per cent of 339 nodular worms were removed from 4 pigs. These animals consumed only 81 per cent of the medicated water within the prescribed period, and none of the pigs were free of large roundworms at necropsy. The recommended dosage of a mixture of piperazine hydrochlorides ‖ was given in about half the daily water consumption to one group of 4 pigs, and 36 per cent of 47 ascarids and 36 per cent of 1,987 nodular worms were removed from the 4 animals; the 30 ascarids recovered at necropsy were in 1 pig. This group consumed only 2 qt. (about one third) of the medicated water within 24 hours, and no significant additional amount within the next 24-hour period. When fresh water was given at the end of the period of treatment, however, the pigs consumed 16.0 qt. within a period of only six hours, and 22.5 qt. within 24 hours. All pigs drank greedily and vomited several times within half an hour. In another trial, when the appropriate dose was given in the amount of water usually consumed in one day, 24 per cent of 63 ascarids and 55 per cent of 2,905 nodular worms were removed from the 4 pigs; all 48 unaffected ascarids were found in 1 pig. This group consumed nearly half of the medicated water within six hours, about 65 per cent within 24 hours, and all within 48 hours. Water consumption was increased about 25 per cent during the 24-hour period following medication, but none of the pigs vomited.

There was no demonstrable action against red stomach worms, thick stomach worms, or whipworms in any of these trials. Treatment was wholly ineffective against 45 Ascarops and only 10 per cent of 1,464 whipworms were removed. No attempt was made to recover Hyostrongylus in the feces after treatment, but an average of 59 per pig was found at necropsy.

DISCUSSION

Published data and that presented herein clearly show that a dosage of 50 mg. of piperazine per pound of body weight is markedly effective against ascarids and nodular worms in individually treated swine. The evidence is less convincing, however, that a similar dosage is equally effective under field conditions. The performance against both helminth species appears to be substantially impaired, in most cases, when the chemical is given by currently accepted practices to groups of as few as 3 or 4 animals. The supportive data are admittedly more suggestive than conclusive, but they indicate the need for a definitive appraisal of the efficiency of the chemical when used under practical farm conditions.

In our limited experience, the lower aggregate efficacy was generally ascribable to 1 or 2 animals of a group; this may have been occasioned by their inability to obtain a proportionate share of the medicated feed or water, or by differences in palatability. Moreover, there was some indication that there may be differences in the latter respect among the several piperazine compounds used. Much for information will be needed to establish this point and to determine the superiority of one product over another. It is recognized that there are occasional failures with most treatments similarly used, and that this need not necessarily militate against using these chemicals in parasite control programs. However, if the proportion of

* The piperazine citrate was supplied by Burroughs Wellcome and Co., Tuckahoe, N. Y.

† The piperazine dihydrochloride was supplied by the Dow Chemical Co., Midland, Mich.

‡ The piperazine sulfate was supplied by Dr. Hess and Clark, Inc., Ashland, Ohio, and by Dr. Salsbury's Laboratories, Charles City, Iowa.

‖ This is a commercial product that was supplied confidentially.

failures with piperazines is maintained in groups larger than those used to date, the practical limitations of the treatment would be obvious. Additional critical studies with groups of 10 or more animals are indicated.

A dosage larger than 50 mg. per pound of body weight may be necessary when the chemical is given in limited amounts of feed or water to groups of pigs. In a field evaluation of swine ascaricides, Kelley *et al.*[5] obtained excellent results with piperazine adipate, although they used the chemical in amounts exceeding 100 mg. of piperazine per pound of body weight, or at a rate approximately twice that generally recommended. Occasional failures attributable to differences in palatability may necessitate the development of suitable flavoring or masking agents, or the search for other salts that are more acceptable.

Piperazine citrate appeared to be the most satisfactory of the several compounds employed in the trials reported herein. When given in drinking water, it was apparently palatable to all animals and was markedly effective against both ascarids and nodular worms. However, because variations in palatability and efficacy were observed in different trials with the several piperazine compounds, it would not be advisable to attach undue significance to the performance of the chemical in this single instance. For example, when piperazine sulfate was used, all large roundworms and the lone nodular worm were removed in one trial, in which the entire amount of medicated water was consumed within the relatively short period of seven hours; the efficiency against both helminth species was significantly lower when only 85 per cent of the medicated water was consumed in 24 hours. In other trials as well, the relative palatability of the several preparations seemed to be reflected in the efficiency of the treatments against both ascarids and nodular worms.

Piperazine *per se* does not appear to exhibit significant action against stomach worms. However, inasmuch as carbon disulfide is produced when piperazine-1-carbodithioic acid dissociates in weak acids, such as gastric juice, it has been suggested [7,8] that this product might offer some promise in this regard. Although no critical data were obtained with Hyostrongylus, postmortem worm count data did not suggest that piperazine-1-carbodithioic acid was significantly effective against this species. Moreover, limited critical data showed that none of the derivatives exhibited action against Ascarops.

SUMMARY

Available information shows that piperazine is a safe and reliable agent for the removal of ascarids and nodular worms from swine that consume at least 50 mg. of the chemical per pound of body weight within approximately 24 hours.

Data are presented which indicate that the chemical may not be uniformly effective, when used by currently accepted practices, under field conditions. Competition among pen mates for limited amounts of medicated feed or water, differences in palatability of available preparations, and marked variations in water consumption occasioned by seasonal or diurnal fluctuations in atmospheric temperature may all contribute to inadequate dosing.

The need for critical studies of the several piperazines under practical farm conditions is indicated by the data presented and by the paucity of such information in the published record.

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Electrophoretic Patterns of Blood Serum from Pig Fetuses and Young Pigs

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ELECTROPHORETIC investigations of serum proteins in pigs have been carried out by many workers.[2,4-7,9,13,16,18,23,24,27,28,30] Extensive study of the protein components immediately after birth and during the first period of life seems, however, to have been undertaken only by Rook *et al.*,[27] Köhler,[18] and Staub and Boguth.[28] Serum from pig embryos has been examined electrophoretically by Moore *et al.*[25]

This article describes the examination by paper electrophoresis of serum from pig fetuses and young pigs, both normally and artificially raised, during the first three months of life. The investigation was designed partly to study the different protein components, especially γ-globulin, at various times, and partly to compare these components in normal suckling pigs and artificially raised pigs.

MATERIALS AND METHODS

Two groups of pig fetuses were examined. One group consisted of 9 fetuses, approximately 2½ months old, and the other of 12, approximately 3½ months old. The uteri were obtained from the Stockholm municipal abattoir and originated from Swedish Landrace sows.

Twelve newborn pigs from three Swedish Landrace litters were examined before being allowed to nurse. Blood samples were subsequently taken from the pigs of one litter, 24 and 48 hours after birth. At this time, the sow developed agalactia, and the pigs were transferred to a battery and raised by the methods of Dyrendahl *et al.*,[8] using a synthetic milk diet composed of dried skimmed milk, lard, rapeseed oil, sugars, yeast, lecithin, essential minerals, vitamins, and antibiotics. When the pigs were about 6 weeks of age, solid feed consisting of crushed oats, wheat bran, meat meal, fish meal, and minerals was given and they were transferred from the battery to a box. Blood samples were taken weekly or bi-weekly until the pigs were 13 weeks old.

Blood samples were taken from normally raised Swedish Landrace pigs from three breeding farms at 2, 4, 7, 8, and 12 weeks of age. Those in each age group were litter mates (table 4).

Blood was obtained from the hearts of the fetuses and the pigs by cardiac puncture (up to 1 week of age). Blood samples were taken from the anterior venae cavae through the thoracic apertures of older pigs. Immediately after coagulation, the blood was centrifuged and the serum stored at −60 C.

Matthews and Buthala[20] have recently shown that when pig serum is frozen a precipitate often appears, but no significant changes in the amounts of protein have been seen in spite of deep freezing over long periods.

Albumin and α-, β-, and γ-globulin have been determined by paper electrophoretic methods, chiefly by those of Kunkel and Tiselius.[17]

Diethylbarbituric acid buffer (sodium diethylbarbiturate, 21.9 Gm., and diethylbarbituric acid, 7.4 Gm., in 1,000 ml. distilled water; pH = 8.5) was used, and the period of electrophoresis was 17 hours under 180 v., with a current of 3 ma. for each strip. Whatman No. 1 paper strips, 4 cm. wide, were used, with 0.02 ml. of serum on each strip. Bromphenol blue (1%) in absolute alcohol was used for staining. The stain was subsequently eluted from strips 3 mm. in width by Na_2CO_3 (1%) in methanol. The intensity of the color was determined in a Beckman B photometer at 5,950 Å. Two electrophoretic separations were carried out on each sample.

Total nitrogen was determined by the method of Miller and Houghton.[22] The standard error (standard deviation of the mean) was calculated by accepted statistical methods.[3]

RESULTS

Electrophoresis of serum from 2½-month-old pig fetuses shows distinct division into three fractions (table 1). In one instance, a division of the most mobile fraction into two secondary fractions of equal size could be observed. In general, the fractions appeared to be homogeneous. These fractions took up positions corresponding to those of albumin and α- and β-globulin in serum from newborn and older pigs.

In serum from 3½-month-old fetuses, a similar division into three distinctly separate fractions, albumin, α-globulin, and β-globulin, was detected (table 1). The fraction which was designated α-globu-

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TABLE 1—Relative Percentages of Protein Fractions in Serum from Pig Embryos and Newborn Pigs Before Suckling

Animals	Albumin	α-globulin	β-globulin	γ-globulin
9 pig embryos, 2½ months' gestation	24 ± 1.9*	42 ± 2.3	34 ± 1.4	0
12 pig embryos, 3½ months' gestation	17 ± 1.0	68 ± 2.2	15 ± 1.8	0
12 newborn pigs, before suckling	13 ± 0.8	68 ± 2.2	19 ± 1.8	0

* Standard error.

lin was especially accentuated and, in approximately half of the cases, the albumin was further subdivided into secondary fractions. Of these, the more mobile fraction was always the smaller.

The electrophoretic separation of serum from newborn pigs which had not nursed did not demonstrate any marked deviations from results obtained with serum from 3½-month-old fetuses (table 1). Again, the α-globulin fraction was the largest. In practically every case, a further division of the albumin fraction into two secondary fractions was seen, with the more mobile fraction being the smaller. In the analysis, these fractions were treated as one, *i.e.*, as the albumin fraction. In serum from this group, as in serum from the group of fetuses, no γ-globulin could be observed.

When pigs reached 1 day of age (following nursing), an important change in the composition of the blood serum was noticed (table 2). Doubling of the total N values, a large increase in β-globulin, and the appearance of large amounts of γ-globulin could be demonstrated (tables 2, 3). An increase in the albumin level could also be detected (table 3). Division of the albumin fraction was less perceptible in these cases. The proportion of α-globulin in the total protein had greatly decreased (table 2) but, if this is considered in conjunction with the increased level of total protein, the actual decrease in the amount of α-globulin was insignificant (table 3).

The serum proteins derived from the colostrum, chiefly in the β-globulin and γ-globulin fractions, seemed to be fairly heterogeneous, electrophoretically. As a result, the various globulin fractions in 1-day-old pigs were indistinctly separated from one another, and it was, therefore, difficult to determine the amounts of the various fractions. In addition, the fractions had wide individual variations (table 2). Stabilization occurred fairly rapidly and, as early as two days after birth, the fractions were easily distinguishable and had greater individual uniformity (table 2).

In serum from 1-week-old pigs, the fractions were distinct, apparently homogeneous, and were individually uniform. In comparison with serum from 1-day-old pigs, it was also possible at this time to observe an increase in the albumin fraction, a decrease in the α-globulin fraction and, on the whole, unchanged values for the β-globulin and γ-globulin fractions of the serum proteins (table 2).

The artificially raised pigs, which were transferred to a battery and placed on the diet proposed by Dyrendahl *et al.*[8] after having nursed for two days, developed diarrhea at about 14 days of age. It was controlled by changing the diet but recurred and gradually became worse on the solid food. The rate of growth decreased and, one week after the last blood samples were taken (when pigs were 3 months old), 2 pigs died and 3 were killed. Pronounced

TABLE 2—The Relative Percentages of Protein Fractions and Total N (in Per Cent) in Serum from Pigs Immediately After Birth

Animals	Albumin	α-globulin	β-globulin	γ-globulin	Total N (%)
12 newborn pigs, before suckling	13 ± 0.8*	68 ± 2.2	19 ± 1.8	0	0.38 ± 0.035
4 pigs, 1 day old	13 ± 0.9	29 ± 4.8	33 ± 3.2	25 ± 3.7	0.78 ± 0.061
4 pigs, 2 days old	13 ± 0.7	30 ± 2.3	32 ± 2.6	26 ± 1.1	.....
5 pigs, 1 week old	25 ± 0.9	22 ± 1.5	29 ± 1 4	24 ± 1.7	.....

* Standard error.

TABLE 3—Percentages of Total Protein, Albumin, α-Globulin, β-Globulin, and γ-Globulin in Serum from Newborn and 1-Day-Old Pigs

Animals	Albumin	α-globulin	β-globulin	γ-globulin	Total protein
12 newborn pigs, before suckling	0.31	1.62	0.45	0	2.40
4 pigs, 1 day old	0.63	1.41	1.61	1.22	4.87

The values for albumin, α-globulin, β-globulin, and γ-globulin have been calculated from the averages for the relative percentage values and the average values (in per cent) for total N ($N \times 6.25 =$ total protein).

ileitis and colitis were found in all pigs at necropsy.

The relative percentages of the protein fractions and of total N (in %) at various times, in serum from pigs raised both normally and artificially, is compared (table 4), and a schematic comparison between the actual concentrations of albumin, α-globulin, β-globulin, and γ-globulin is given (fig. 1).

The values for total N in the serum of normally raised pigs rose during the experimental period. In serum from artificially raised, 4-week-old pigs, the total N levels were only insignificantly lower than in serum from normally raised pigs. Toward the end of the experimental period, however, the total N levels in the serum of the artificially raised pigs decreased to about half the values for serum from normally raised pigs (table 4).

The relative levels of albumin were maintained at practically the same levels in serum from pigs in both groups up to the time when the pigs were between 8 and 13 weeks of age (table 4). The relative, as well as the absolute, albumin levels reached a maximum in the samples taken from pigs of both groups at 4 weeks of age and then, in serum from pigs in the normally raised group, decreased to values which agree with those found by other investigators for serum from mature pigs.[5,7] The absolute values for albumin in serum from pigs in the artificially raised group were, however, significantly lower than in serum from pigs in the normally raised group as early as 4 weeks of age (fig. 1). This difference between pigs of the two groups was further accentuated by the lower total protein values and the greater decrease in the relative amounts of albumin in serum from artificially raised pigs (table 4).

The relative levels of α-globulin in the serum seemed to reach a minimum in pigs of both groups at 4 weeks of age. In spite of the wide differences in the relative amounts seen in the serum of pigs in the two groups at the final blood sampling (table 4), there were, apparently, no noteworthy differences in the actual amounts (fig. 1).

Approximately similar results were obtained for β-globulin. After the sharp increase in β-globulin originating from colostrum, the β-globulin levels progressively declined until, in the serum of the normally raised but not the artificially raised pigs, the levels were again found to be increasing when the pigs reached 8 weeks of age (fig. 1).

Minimum values for γ-globulin in the serum were obtained when the pigs were 4 weeks of age. Then, the levels rose progressively, although the rate of increase was slower (table 4) and the ultimate values were not as great in the serum of artificially raised pigs as they were in the serum of normally raised pigs (fig. 1).

DISCUSSION

Investigations of the protein components in the serum of pig embryos at different stages of development have been previously carried out by Moore *et al.*,[25] who were able to demonstrate changes in the types and amounts of proteins during embryonic development.

The protein substances, derived from colostrum and later from synthesis, which form the serum proteins of young pigs are probably not all of the same type as those which can be found in the serum immediately after birth. The protein fractions in the serum of pig embryos, newborn pigs, and older animals may differ in composition, although the various fractions apparently correspond to one another electrophoretically. The fractions which, electrophoretically, resemble albumin and α-and β-globulin in serum from pig fetuses and newborn pigs have been designated, for the sake of convention, albumin and α- and β-globulin.

The relative proportions of the various protein fractions appear to undergo changes during fetal development from 2½ to 3½ months of gestation. The pro-

portion of albumin is somewhat lower and that of β-globulin is more than 50 per cent lower in the serum of 3½-month-old fetuses than in the serum of 2½-month-old fetuses. The proportion of α-globulin, on the other hand, greatly increases from 2½ to 3½ months of gestation. Since only small amounts of blood could be obtained from the fetuses, total protein values could not be determined. It can, however, be assumed that the total protein level in fetal serum probably increases during development and that, during this period, the absolute increase in α-globulin is pronounced and the decrease in β-globulin of significant proportions. It is somewhat uncertain whether changes in the albumin levels occur.

Important changes in the amounts of the protein fractions in the serum apparently do not occur during the period between 3½ months of gestation and birth.

In serum obtained from 300-mm. embryos, Moore *et al.*[25] found a protein fraction which occupied a position corresponding to the γ-globulin fraction in the serum of older pigs. A similar fraction has not been observed during the course of the present experiments.

In agreement with the work of others,[14,18,21,27,28] no γ-globulin could be demonstrated in the serum of newborn pigs before nursing. This is also true for the serum of calves[15] and foals.[26] Earlier observations that large quantities of γ-globulin,[14,18,27,28] β-globulin, and somewhat less albumin (but no α-globulin[27]), appear in the serum soon after the administration of colostrum could be confirmed.

An unidentified protein fraction, "component X," lying between the albumin and α-globulin fractions, has been observed in electrophoretic examinations of serum from newborn pigs by Rook *et al.*[27] This fraction rapidly decreases in amount, and can not be definitely identified after pigs have reached 5 days of age. A similar fraction has not been observed in the present investigation. It is possible that this deviation in results may be accounted for by the different methods of electrophoresis which have been used. The component X described by Rook *et al.*[27] may have been dispersed in the present experiments, so that some of it formed the less mobile portion of the divided albumin fraction (observed in serum from newborn pigs), while the rest was included in the α-globulin fraction. Another possibility is that, in the present experiments, component X was completely included in the α-globulin fraction, and that the more mobile portion of the albumin fractions may correspond to the new component described by Malmnäs and Nihlén[19] in human fetuses as migrating ahead of the albumin.

Rook *et al.*[27] and the present writer found that α-globulin formed the largest protein fraction in the serum of newborn pigs. However, Köhler[18] found the β-globulin fraction to be the largest, with values corresponding approximately to those for the α-globulin fraction in the present experiments and the component X plus α-globulin found by Rook *et al.*[27] A possible explanation for this discrepancy is that Köhler[18] used relatively high voltage in his electrophoretic experiments.

The presence of a new protein substance, fetuin, with an electrophoretic mobility between that of α-globulin and β-globulin, has been demonstrated in the serum of newborn calves by Adair and Pedersen[1] and, later, by Hansen and Phillips.[12] On the basis of ultracentrifuge experiments, Moore *et al.*[25] considered the possibility that a fetuin-like substance does occur in the serum of pig embryos. In none of the electrophoretic examinations carried out on serum from pig fetuses and newborn pigs during the course of the present experiments could the presence of this substance be observed.

When pigs are approximately 1 month old, the albumin levels in the serum reach a maximum, while all the globulin fractions drop to minimum levels (fig. 1). *Gamma*-globulin is of special interest in this connection, since most of the anti-

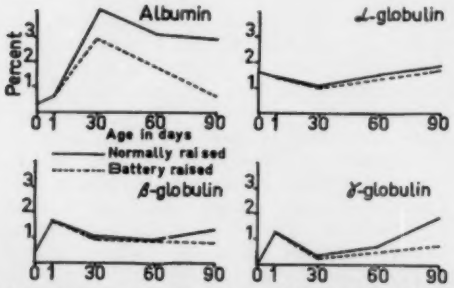

Fig. 1—Concentration of protein components in the serum of normally and artificially raised pigs; calculated from the average values for relative percentages of the various components and the average values (in per cent) for total nitrogen (N × 6.25 = total protein).

TABLE 4—Relative Percentages of Protein Fractions and Total N (in Per Cent) in Serum from Pigs of Various Ages, Both Normally and Artificially Raised

Animals (No.)	Serum from pigs raised normally					Animals (No.)	Serum from pigs raised artificially					
	Albumin	α-globulin	β-globulin	γ-globulin	Total N (%)		Albumin	α-globulin	β-globulin	γ-globulin	Total N (%)
4 pigs, 1 day old	13 ± 0.9*	29 ± 4.8	33 ± 3.2	25 ± 3.7	0.78 ± 0.061	4	13 ± 0.9	29 ± 4.8	33 ± 3.2	25 ± 3.7	0.78 ± 0.061
5 pigs, 2 weeks old	46 ± 2.8	19 ± 1.3	25 ± 1.6	10 ± 1.3	.....	6	38 ± 3.3	26 ± 1.9	22 ± 1.9	14 ± 2.0	.....
5 pigs, 4 weeks old	61 ± 2.7	17 ± 1.0	16 ± 1.0	6 ± 1.0	1.02 ± 0.085	5	57 ± 1.2	20 ± 1.4	18 ± 0.6	5 ± 0.6	0.82 ± 0.022
5 pigs, 7 weeks old	48 ± 1.8	23 ± 1.7	18 ± 0.8	11 ± 0.6	.....	5	46 ± 3.9	26 ± 3.5	21 ± 2.2	7 ± 0.4	.....
5 pigs, 8 weeks old	49 ± 2.9	25 ± 2.0	14 ± 0.4	12 ± 0.4	1.02 ± 0.028	5	44 ± 1.7	29 ± 3.0	21 ± 1.6	6 ± 0.5	.....
4 pigs, 12–13 weeks old	37 ± 1.0	23 ± 1.6	17 ± 1.5	23 ± 1.6	1.29 ± 0.067	5	17 ± 3.5	43 ± 2.2	21 ± 1.6	19 ± 2.5	0.64 ± 0.089

* Standard error.

bodies found in the blood are concentrated in this fraction.[10, 11, 31] After the ingestion of colostrum, the levels of γ-globulin in the serums reach a peak, followed shortly by a progressive decline until, when pigs are 4 weeks old, it comprises only 5 to 6 per cent of the total protein. At this time, the synthesis of γ-globulin first seems to reach such proportions that the progressive decline in the levels of the γ-globulin passively transferred from the colostrum can be covered and exceeded.

The time at which the levels of γ-globulin in the serum reach minimum values corresponds to the most sensitive period for the health of pigs. This association supports the hypothesis that the parenteral administration of γ-globulin could be of assistance in the treatment or prevention of infections in pigs of this age (4 weeks).

Diarrhea, usually attributed to overfeeding,[8] occasionaliy occurs in artificially raised pigs. A change of feed usually has a favorable effect in such cases. The diarrhea which occurred in the artificially raised pigs at 14 days of age was quickly controlled by similar measures. When the pigs were given solid feed, diarrhea recurred but, in order to observe the states of the protein components under extremely unfavorable conditions, no serious attempt was made to control it.

Until the pigs reached 4 weeks of age, while the artificial raising progressed normally, the total protein in the serum from pigs of this group was, on the average, about 1.3 Gm./100 ml. (approx. 20%) lower than in the serum from pigs of the normally raised group. The difference is almost entirely dependent upon the lower (approx. 1 Gm./100 ml.) albumin level in the serum from pigs of the artificially raised group.

The average protein levels, in the serum of the artificially raised pigs at 3 months of age, were 4.1 Gm./100 ml. (approx. 50%) lower than in serum of normally raised pigs, a finding which appears to be reasonable in view of the violent diarrhea. This difference also depended largely on a lower albumin level, as well as on a somewhat smaller decrease in the β- and γ-globulin levels. Depression of the α-globulin level was insignificant (fig. 1). While the observation of a continuously lower albumin level is of interest, many more examinations would be required to

ascertain whether this change represents the actual picture in the serum of artificially raised pigs and, if so, whether it is of any significance.

Staub and Boguth[28] have recently shown that pigs which have not received colostrum begin to synthesize γ-globulin at 2 weeks of age and that, by 8 weeks of age, the levels of γ-globulin in the serum of such pigs closely agree with the values obtained for serum of pigs which have received colostrum. The slower rate and lesser extent of γ-globulin synthesis in pigs of the artificially raised group, as compared with pigs of the normally raised group, in the present experiment, is probably associated with the diarrhea in that group.

SUMMARY

Serums from pig fetuses, newborn pigs before and after nursing, and pigs raised under normal and artificial conditions for the first three months of life have been examined by paper electrophoresis.

Three distinct protein fractions, albumin, and α- and β-globulin could be detected in serum from pig fetuses of 2½ and 3½ months' gestation and from newborn pigs. Differences in the proportions of the fractions between 2½ and 3½ months of gestation were observed. Between 3½ months of gestation and birth, the relative values for the various fractions apparently did not undergo major changes.

No γ-globulin could be detected in serum from pig fetuses and newborn pigs. Division of the albumin fraction was observed in the serum from the newborn pigs and from some of the fetuses. Large amounts of γ-globulin appeared in the serum shortly after nursing. In addition, a marked increase in the levels of total N and β-globulin, and a smaller increase in the albumin level, but no change in the α-globulin, occurred at this time.

A continuous increase of the total nitrogen (N) occurred in serum from the normally raised pigs during the course of the experiment. The albumin levels reached maximum values, and the globulin fractions reached minimum levels in serum from 4-week-old pigs. *Gamma*-globulin decreased most and, when the pigs were 4 weeks of age, comprised only 5 to 6 per cent of the total protein in the serum. After this time, the globulin levels increased continuously.

Total N values in the serum from artificially raised pigs increased, initially, and decreased toward the end of the experimental period. In 4-week-old pigs, the levels of absolute albumin in the serum were noticeably lower than in serum from normally raised pigs at the same time, and decreased markedly toward the end of the experiment. The values for β-globulin and γ-globulin in the serum of artificially raised pigs were also lower than those in the serum from pigs of the normally raised group at the end of the experiment. No essential differences in the α-globulin levels in the serum from pigs of the two groups were observed.

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Toxicity of Heated Swine Serum

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MUNCE AND HOFFMAN,[3] in 1930, showed that injecting young, healthy swine with heated hyperimmune anti-hog cholera serum produced shock in the inoculated animals. Shock was most severe when the serum had been previously heated at temperatures from 60 to 62 C. for 30 minutes. They found no similar response when they inoculated swine with an equal quantity of nonheated, hyperimmune anti-hog cholera serum. Phenol, salt, and bean extract were eliminated as contributing factors. Recently, Schipper, Bolin, and Eveleth [8] showed the importance of anemia as a factor contributing to reactions following vaccination. These workers noticed that swine with hemoglobin levels of 7 Gm., or less, per 100 ml. of blood are predisposed to vaccination reactions of marked severity resulting, in some cases, in death.

In a previous paper, Buthala and Mathews [1] showed that commercial pasteurization, at 58.5 C., of hyperimmune anti-hog cholera serum resulted in a modification of the electrophoretic mobility of certain serum proteins. This modification was reflected in the formation of an albumin-*gamma* globulin complex (AGGC) which migrated electrophoretically in the *alpha-beta* globulin zone. Although the percentage of unmodified *gamma* globulin was reduced, the serum retained its effectiveness in immunization of swine against hog cholera virus.

This investigation was conducted to determine the relationship of AGGC to postvaccinal shock in swine and to relate the toxicity following inoculation of swine with heated serums to the temperature at which the serums are heated.

MATERIALS AND METHODS

A pool of nonimmune swine serum was obtained from 4 nonvaccinated animals, 4 months old. A hog cholera immune serum pool was obtained from 2 swine, 8 months old, three months after vaccination with serum and live virus. Three hyperimmune anti-hog cholera serum pools, collected prior to pasteurization and phenolization, were obtained from a commercial source.

Pasteurization, at various temperatures, was accomplished by immersing a wide-bottomed flask containing a shallow layer of serum in a water bath. Approximately five minutes were required to bring the serum to the desired temperature. All samples were maintained at a given temperature, $\pm$ 0.5 of a degree C. for 30 minutes and then immediately chilled in running tap water unless otherwise indicated.

Methods of electrophoretic analysis and separation of AGGC have been described previously.[2] The test animals used were nonvaccinated swine, 30 to 40 lb. Inoculations were made intraperitoneally unless otherwise indicated.

RESULTS AND DISCUSSION

Effect of Heating on the Protective Properties.—To evaluate the effect of various temperatures on the protective properties of hyperimmune anti-hog cholera serum, a quantity obtained prior to pasteurization and phenolization was divided into five 57-ml. portions. To each portion was added an equal volume of physiological saline solution to prevent coagulation at high temperatures. After heating for 30 minutes at 59, 62, 65, 70, or 75 C., these samples were used with commercial hog cholera virus-containing blood to vaccinate 200-lb. swine. The temperature curves of the inoculated animals are shown (graph 1). The animal which received the serum heated at 75 C. succumbed to hog cholera infection. The other 4 animals were protected and, when challenged, were shown to be immune. It is evident that temperatures of 70 C. or less do not completely destroy the protective antibodies of anti-hog cholera serum. Thus, it might be concluded that pasteurization, as practiced commercially, does not markedly alter the protective antibodies of hyperimmune serum, although prolonged exposure to temperatures as low as 58 C. does modify certain of the serum components.

Effect of Heating on Production of the Albumin-Gamma Globulin Complex.—Electrophoretic analyses of nonheated and heated samples of nonimmune serum and immune and hyperimmune anti-hog cholera

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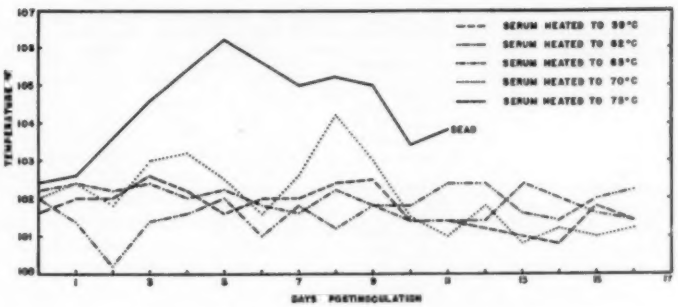
This work was supported in part by a grant from the Associated Veterinary Laboratories, Inc.



Graph 1—Temperature curves of swine vaccinated with heated, hyperimmune anti-hog cholera serum and blood containing hog cholera virus.

serums showed that, as the temperature of treatment was increased, there was an increase in AGGC with a corresponding decrease in unmodified albumin and *gamma* globulin (table 1).

The electrophorograms of heat-treated nonimmune serum are shown (graph 2). Comparable results were obtained with heat-treated immune and hyperimmune anti-hog cholera serums. The sample of nonimmune serum which was treated in a manner simulating commercial pasteurization, *i.e.*, 30 minutes heating to reach the desired temperature, 30 minutes at pasteurization temperature (58.5 C), and 15 minutes to cool, had an electrophoretic pattern corresponding to an experimental pasteurization temperature of 59 to 60 C.

Toxicity of Heated Whole Serums.—Intraperitoneal inoculation of swine with heated whole nonimmune or immune anti-hog cholera serum (table 2) showed that only those samples that were heated at either 62 C. or 65 C. produced postinoculation reactions in the test animals. These reactions were observed within 30 minutes after administration. Moderate to severe reactions were noticed. Moderate reactions were recorded if the animal showed hyperemia of ears and snout, depression, slight posterior incoordination, and mild dyspnea, with the signs lasting for one hour or more. Severe reactions were recorded for animals showing pronounced posterior incoordination, marked dyspnea, frequent vomiting, extreme depression, and intense

TABLE 1—Electrophoretic Analysis of Heated Swine Serums

Type of serum	Treatment	Percentage of total area				
		Albumin	*Alpha*	*	*Beta*	*Gamma*
Nonimmune	Nonheated control	47.5	17.5	.....	16.7	18.3
	58 to 59 C.	42.9	.....	39.8 (05.6)	.....	17.3
	60 to 61 C.	39.1	.....	46.7 (12.5)	.....	14.2
	62 to 63 C.	27.3	.....	58.5 (24.3)	.....	14.2
	64 to 65 C.	20.7	.....	70.5 (36.3)	.....	8.8
	Slow heat †	40.1	.....	42.7 (08.5)	.....	17.3
Immune	Nonheated control	‡	.....	.....	14.1	16.8
	55 to 56 C.	48.2	20.9		14.1	16.8
	58 to 59 C.	42.7	.....	42.3 (07.3)	.....	15.0
	60 to 61 C.	37.5	.....	47.5 (12.5)	.....	15.0
	62 to 63 C.	29.4	.....	57.3 (22.3)	.....	13.4
	64 to 65 C.	23.0	.....	66.6 (31.6)	.....	10.4
Hyperimmune 1	Nonheated control	33.9	24.0	.....	17.6	24.4
	58 to 59 C.	28.2	.....	50.1 (08.5)	.....	21.6
	60 to 61 C.	27.2	.....	50.6 (09.0)	.....	22.2
	62 to 63 C.	19.5	.....	61.2 (19.6)	.....	19.3
	64 to 65 C.	22.2	.....	60.7 (19.1)	.....	17.1
Hyperimmune 2	Nonheated control	34.6	21.2	.....	15.7	28.4
	58 to 59 C.	32.9	.....	44.1 (07.2)	.....	23.0
	60 to 61 C.	29.9	.....	50.3 (13.4)	.....	19.8
	62 to 63 C.	‡	.....	.....	.....	.....
	64 to 65 C.	22.9	.....	58.7 (21.8)	.....	18.4

* *Alpha* and *beta* globulins not resolvable. Number in parentheses is percentage of albumin-*gamma* globulin complex. Example: $39.8 - (17.5 + 16.7) = 5.6$.

† Thirty minutes to reach temperature, 30 minutes at 58 to 59 C. and 15 minutes to cool (simulates commercial pasteurization).

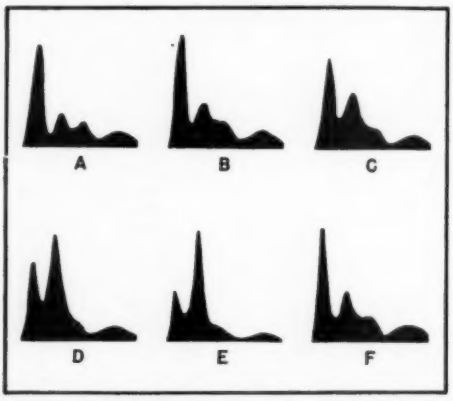
‡ Sample lost during dialysis prior to electrophoresis.

TABLE 2—Inoculation of Swine with Whole, Heated Serums

Type of serum	Treatment	No. of animals	Weight (lb.)	Inoculum (ml.)	Reaction
Nonimune	Nonheated control	1	34	35	None
	58 to 59 C.	1	37	35	None
	60 to 61 C.	1	35	35	None
	62 to 63 C.	1	33	35	Moderate
	64 to 65 C.	1	34	35	Severe
Immune	Nonheated control	1	34	35	None
	55 to 56 C.	1	36	35	None
	58 to 59 C.	1	32	35	None
	60 to 61 C.	1	36	35	None
	62 to 63 C.	1	38	35	Moderate
	64 to 65 C.	1	34	35	Severe
	68 to 69 C.	1	41	35	None

hyperemia of ears, snout, and underline, with the signs persisting for one hour or more. In both moderate and severe reactions, anorexia was observed for one to three days. The sample of serum heated at 68 to 69 C. coagulated into a viscous gel and did not produce a reaction. Postinoculation reactions were not noticed in animals injected with whole serum heated at temperatures below 62 C.

Toxicity of Heat-Produced AGGC *and Heated Serums Freed of the Complex.*—The AGGC separated from various samples of heated whole immune anti-hog cholera serum, when injected intraperitoneally into swine (table 3), produced a response similar to that shown following administration of the heated whole immune serum (table 2). No reaction was observed following inoculation of heated serums which had been freed of AGGC. This indicated that AGGC was the etiological factor in the observed reactions.



Graph 2—Electrophorograms of nonheated and heated nonimmune serum: (A) nonheated, nonimmune serum; (B) heated 58 to 59 C. for 30 minutes; (C) heated 60 to 61 C. for 30 minutes; (D) heated 62 to 63 C. for 30 minutes; (E) heated 64 to 65 C. for 30 minutes; (F) heated 58 to 59 C. (30 min. to reach temperature, 30 min. at 58 to 59 C. and 15 min. to cool).

Although reactions were noticed after both intraperitoneal and intravenous inoculations of the appropriate material, reactions of greater severity and rapidity were noticed using the latter route. It is evident that the route of inoculation plays an important part in these postinoculation reactions.

In the present study, it was not determined whether the increased toxicity of the serum heated to 64 to 65 C. was due to an increase in the amount of AGGC or to a complex which differs from that produced at lower temperatures.

In view of the increased toxicity of serum heated at 62 to 65 C. over serums heated at lower temperatures, and the tendency of increasing amounts of AGGC to form on prolonged heating, the importance of the findings of Ray and Whipple [7] is emphasized. These workers found that a temperature of 60 C. may be attained in the trunks of automobiles during the summer. Prolonged exposure of serum to this temperature may produce toxicity.

Serological and Immunological Relationship of Swine AGGC *and Equine* AGGC.—Pasteurization of equine serum produces an AGGC physically similar to the AGGC of heated swine serum,[9] and which posseses the property of producing shock in the homologous animal.[6] Some field observers suspect that swine, previously injected with anti-erysipelas serum of equine origin and subsequently inoculated with hyperimmune anti-hog cholera serum, may experience severe postvaccinal reactions. In order to investigate the possible serological relationship of the AGGC of equine origin to the AGGC of swine origin, rabbit anti-

serum was prepared against AGGC of swine origin separated from commercial hyperimmune anti-hog cholera serum. By means of the ring-precipitin test, it was shown that this antiserum gave a high titer against AGGC of either swine or equine origin. The antiserum gave a low titer when associated with normal nonheated serums of several horses. No precipitation was noticed when rabbit antiserum, prepared against whole nonheated swine serum, was tested against nonheated equine serum. Further evidence of serological relationship was furnished by absorbing the rabbit-produced anti-swine AGGC serum with AGGC of equine origin. The titer of the absorbed antiserum against the swine AGGC was considerably reduced.

By the method of Oudin [5] as modified by Oakley,[4] it was found that the AGGC of heated equine serum produced two distinct but diffuse bands of precipitation when migrated against rabbit-produced anti-swine AGGC serum. These bands of precipitation were completely inhibited by incorporation of AGGC of either swine or equine origin into the agar of the diffusion system. It is evident from the data that the AGGC of heated equine serum is serologically related to the AGGC of heated swine serum.

In order to determine the potential ability of either swine or equine AGGC to produce shock in other experimental animals, 16 guinea pigs (wt. 200 Gm. each) were divided into four groups of 3 animals and one group of 4 animals (table 4). Each animal of groups A and B was given, intraperitoneally, 1.0 ml. of swine AGGC in a dilution of 1:100. Each animal of groups C and D received 1.0 ml. of equine AGGC in a dilution of 1:100, intraperitoneally, and group E, containing 4 animals, served as a control, each animal receiving 1.0 ml. of saline solution intraperitoneally. Each animal in group A was given 5 ml. of swine AGGC intracordially 21 days after inoculation; all animals died of typical anaphylactic shock. Each animal in group B was given 5 ml. of equine AGGC intracordially; all animals survived but showed definite signs of shock. Group C animals were given 5 ml. of equine AGGC intracordially; all animals died of shock. Group D animals were given 5 ml. of swine AGGC intracordially; all animals died of shock. In group E, 2 animals were given 5 ml. of equine AGGC intracordially and the remaining 2 were given 5 ml. of swine AGGC via the same route. All animals in the control group survived and exhibited no signs of shock. This experiment was repeated with different sources of both swine AGGC and equine AGGC, with essentially the same results. Guinea pigs, sensitized with either normal nonheated equine serum or normal nonheated swine serum, gave no signs of shock when given a potentially shock-producing dose of the heterologous serum.

TABLE 3—Inoculation of Swine with AGGC from Heated, Immune Anti-Hog Cholera Serum

Treatment	No. of animals	Weight (lb.)	Reaction
Nonheated control *	1	34	None
55 to 56 C.	1	38	None
58 to 59 C.	1	36	None
60 to 61 C.	1	32	None
62 to 63 C.	1	36	Moderate
64 to 65 C.	1	34	Severe

* Each inoculum contained the AGGC separated from 32 ml. of serum.

To evaluate this immunogenic relation in swine, each of 5 anemic pigs was inoculated with 5 ml. of commercial equine anti-erysipelas serum, another group of 5 anemic animals remained uninoculated. Each of a third group of 5 normal animals was given 5 ml. of the above mentioned anti-erysipelas serum, and a fourth group of 5 nonanemic animals remained uninoculated. Each animal was inoculated intraperitoneally, 21 days later, with 35 ml. of commercial anti-hog cholera serum. None of the test animals died. However, several of the anemic animals which had been sensitized with the anti-erysipelas serum showed severe shock, remained prostrate for several hours, and failed to eat. Consequently, they lost weight for several days. The anemic animals which were not sensitized showed marked depression and huddling following the inoculation. The normal, nonanemic animals sensitized with

TABLE 4—Anaphylactic Shock in Guinea Pigs Following Administration of Swine or Equine AGGC

Group	No. of animals	First inoculation	Second inoculation	Result
A	3	S *	S	Shock and death
B	3	S	E	Shock
C	3	E	E	Shock and death
D	3	E	S	Shock and death
E	2	Saline solution	E	No shock
	2	Saline solution	S	No shock

* S indicates swine AGGC; E indicates equine AGGC.

anti-erysipelas serum showed shock following administration of the anti-hog cholera serum, although the reaction was not as severe as that noticed in either of the anemic groups. The nonsensitized, nonanemic group showed little evidence of shock.

Schipper, Bolin, and Eveleth [8] found that severe reactions following vaccination were more frequently observed in animals having 7 Gm., or less, of hemoglobin per 100 ml. of blood. The anemic animals used in this study had hemoglobin levels of 9 to 10 Gm./100 ml. of blood. The failure of these animals to show reactions of the severity observed by Schipper, Bolin, and Eveleth [8] may be due to this difference in hemoglobin levels.

SUMMARY

1) Increasing the temperature of pasteurization of swine serum results in a progressive gain in the amount of albumin-*gamma* globulin complex (AGGC) and a corresponding loss of unmodified albumin and *gamma* globulin. This observation is true for nonimmune serums, and for immune and hyperimmune anti-hog cholera serums.

2) Toxicity of heated swine serum is related to the temperature of pasteurization. Toxicity was observed in serums heated at 62 C. and 65 C. but was absent in serums heated at temperatures below 62 C. or at 68 to 69 C. The latter temperature caused the serum to gel.

3) Commercially-produced hyperimmune anti-hog cholera serums contain AGGC. Inoculation of anemic animals with these antiserums may cause reactions of marked severity, while normal animals are not affected.

4) The heat-produced AGGC of swine serum is serologically and immunologically related to the AGGC of heated equine serum.

5) A previous inoculation of anemic or normal animals with anti-erysipelas serum of equine origin may sensitize the animals against a later inoculation with commercial hyperimmune anti-hog cholera serum.

6) The protective antibodies of hyperimmune anti-hog cholera serum survive 30 minutes' heating at 70 C., but are destroyed, or at least markedly reduced, by heating at 75 C. for 30 minutes.

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Effect of Cortisone on the Mechanism of Anaplasma Immunity in Experimentally Infected Calves

I. Hematological and Immunoserological Studies

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ANAPLASMOSIS has been recognized for nearly 50 years as a disease of major economic importance to the cattle industry throughout the tropical and subtropical world. Within recent years, the geographical range of the disease has gradually extended into the temperate zones. Although anaplasmosis has been under investigation for many years, there is still a need for development of basic information on the nature of the causative agent and the phenomena of its complex interactions with the host animal. A characteristic of anaplasmosis is the development of a state of premunition, manifested by persistence of the agent and quiescent infection in cattle that survive the initial attack. While Anaplasma-premune cattle may serve as foci of infection for spreading the disease, such animals are normal, from all outward appearances. These carrier cattle can not be clinically differentiated from noninfected animals, and Anaplasma usually can not be demonstrated in blood films prepared and stained in accordance with presently used methods.

The purpose of the present study was to investigate the possibility of reactivating dormant infection, by the administration of cortisone, to the point that Anaplasma might be demonstrated by microscopic examination of stained peripheral blood smears. The exact mechanism by which cortisone depresses resistance to an infection is unknown; however, it is thought that its action on the mesenchymal tissue, with the resulting depression of inflammatory cell production, is responsible for the effect. With this in mind, the effect of cortisone on experimental bovine anaplasmosis was studied, with the expectation of depressing the protective forces which, in turn, would lead to relapses of the infection. The results were somewhat different than anticipated, and observations were made which contribute to better understanding of the probable mechanism of the infection, with special reference to the protective splenic and extrasplenic cellular and humoral mechanisms and their interaction with the agent.

This report presents the hematological and immunoserological manifestations in cortisone-treated and noncortisone-treated calves which were experimentally infected with Anaplasma.

MATERIALS AND METHODS

Guernsey bull calves (19), 4 to 6 months old, were allotted to five groups for this experiment, with 4 animals in group 2, 6 in group 5, and 3 in each of the remaining three groups. Group 5 was composed of noninfected animals, while animals in the other groups were experimentally infected Anaplasma carriers. The original source of Anaplasma was pooled blood samples, secured from cattle infected with *Anaplasma marginale*, and serially passaged at various intervals during the past several years. The inoculum for these experiments was obtained from an experimentally infected 8-month-old calf, 20 days after splenectomy. The animals were inoculated simultaneously with intravenous inoculations of 50 ml. of heparinized bovine blood, with 70 per cent of the erythrocytes parasitized with one or more Anaplasma bodies. Cortisone* was administered intramuscularly in amounts of 1 mg. per pound of body weight daily, for varying periods (table 1). Surgical removal of the spleen was performed on experimental animals to correspond with the protocol outlined (table 1). The test animals were held

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* Cortone acetate, Merck Sharp and Dohme, Philadelphia, Pa.

TABLE 1—Protocol of Cortisone Treatment of Calves Experimentally Infected with Anaplasma

Status	Group	Animal No.	Splenectomized (days postinfection)	No. of days cortisone* administered: Before splenectomy	No. of days cortisone* administered: After splenectomy
Infected	1	1	42	No cortisone, infected control	
		2	66		
		3	49		
	2	4	52	31	....
		5	42	24	....
		6	49	33	....
		7	46	28	....
	3	8	49	28	35
		9	52	35	35
		10	59	32	28
	4	11	70	....	21
		12	56	....	22
		13	49	....	18
Noninfected	5	14	60	....	....
		15	54	....	....
		16	48	30	....
		17	52	26	19
		18	64	....	14
		19	....	Nonsplenectomized	

* Cortisone administered intramuscularly, 1 mg. per pound of body weight, daily.

under controlled conditions in a screened, insect-proof isolation building, in individual stalls of galvanized iron panels set in concrete floors.

The observation period was limited to a maximum of 65 days after splenectomy. Blood samples for serological studies were obtained two weeks prior to and immediately before infection, and at approximately three-day intervals during the observation period. The complement-fixation technique was essentially that described by Gates *et al.*[3] Serums were stored at —40 C. and were treated two days prior to use with 0.5 per cent phenol. Antigen used in the complement-fixation test was received from the Viral and Rickettsial Disease Section of the U. S. Agricultural Research Service. The complement consisted of fresh, pooled guinea pig serum, preserved by storage at —70 C. in sealed, 2-ml. ampules. The sheep erythrocytes, secured from a local flock, were washed and standardized to a 5 per cent concentration.

Heparinized blood samples were used for hematological studies. The procedures outlined by Gradwohl[4] were used for total leukocyte, erythrocyte, and differential granulocyte counts, and hematocrit determinations. The icteric index was determined by a Leitz-Rouy photoelectric colorimeter, and the technique recommended in the Leitz handbook.* Blood films stained with Giemsa's stain were used to observe and enumerate the Anaplasma bodies. For enumeration of reticulocytes, the methods of Cook-Meyer-Tureen[4] and new methylene blue N stain were used. Since only slight reticulocyte response was usual, performance of the stains was ascertained by using human blood as control.

* E Leitz Co. Handbook for Rouy-Photometer, S-53, New York.

TABLE 2—Effect of Cortisone on Hematological Responses of Calves Experimentally Infected with Anaplasma

	Group 1 No cortisone (infected control)	Group 2 Cortisone before splenectomy	Group 3 Cortisone before and after splenectomy	Group 4 Cortisone after splenectomy	Group 5 No cortisone (noninfected control)
BEFORE SPLENECTOMY					
Packed cell volume (erythrocyte)	27.0% (24–30)*	30.0% (28–32)	29.0% (25–34)	35.0% (30–40)	34.0% (29–38)
Hemoglobin (Gm./100 cc. of blood)	7.5 (6.5–8.6)	8.6 (8.0–9.2)	7.8 (6.8–8.8)	8.2 (5.0–10.2)	9.8 (9.2–10.2)
Red blood cells (millions/cmm.)	4.3 (4.0–4.6)	4.7 (4.3–5.3)	4.8 (3.8–5.8)	4.4 (2.2–8.6)	7.3 (7.0–7.8)
Reticulocyte count	0	0	0	0.7%	0
Granulocyte count	17.0% (13–20)	44.0% (30–40)	43.0% (41–45)	40.0% (31–49)	39.0% (30–47)
Lymphocyte count	83.0% (73–93)	56.0% (50–60)	57.0% (55–59)	60.0% (50–65)	61.0% (53–70)
AFTER SPLENECTOMY					
Packed cell volume (erythrocyte)	17.0% (10–24)	23.0% (20–26)	24.0% (19–29)	16.0% (10–21)	38.0% (35–40)
Hemoglobin (Gm./100 cc. of blood)	4.0 (2–6)	5.9 (5.0–6.8)	6.0 (5.5–6.6)	3.5 (1.5–5.5)	9.4 (9.0–10.0)
Red blood cells (millions/cmm.)	2.9 (2.3–4.0)	3.9 (3.7–4.1)	4.4 (3.8–5.0)	2.5 (1.3–4.0)	7.6 (6.0–9.0)
Reticulocyte count	0	0.6% (0.1–1.6)	1.5% (0.3–3.5)	0	0
Granulocyte count	73.0% (56–90)	51.0% (39–65)	58.0% (50–70)	59.0% (48–70)	45.0% (27–62)
Lymphocyte count	27.0% (19–44)	49.0% (25–58)	42.0% (39–48)	41.0% (29–52)	55.0% (38–73)

* Average and range during a period of three weeks before and after splenectomy.

RESULTS

Data pertaining to the course of infection of animals in four experimental groups, as measured by the percentages of parasitized erythrocytes, hematocrit levels, and complement-fixing antibody responses, are shown (fig. 1–4). Hematological responses of experimental calves, including the Anaplasma-free animals, in average and range during the three-week period before and after splenectomy, are presented (table 2).

Group 1.—The 3 animals in this group served as infected controls and received no cortisone. Low percentages of infected erythrocytes, not exceeding 1.5 per cent, were observed during the presplenectomy period. Hematocrit levels, averaging 30 per cent before infection, receded to 16 per cent at the end of the second week after infection. Hematocrit levels then gradually increased to an average of 27 per cent during the three weeks prior to splenectomy. A complement-fixing antibody response was demonstrated seven days following infection, in serum dilutions of 1:80 and 1:160 and, with slight variations, persisted at this level until splenectomy.

Immediately after splenectomy, there was a sudden increase of infected erythro-

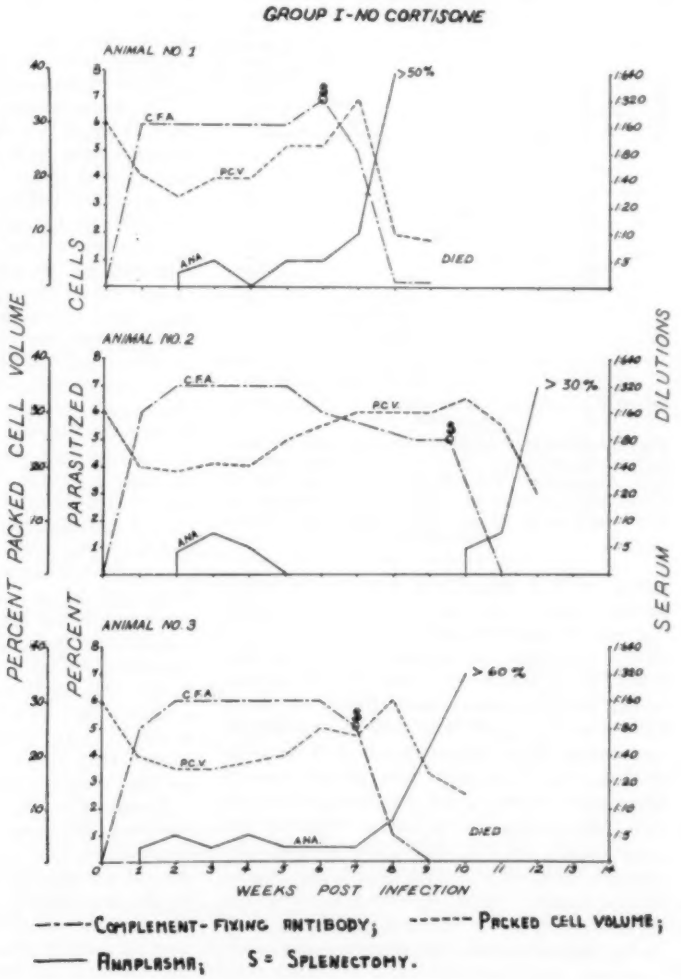
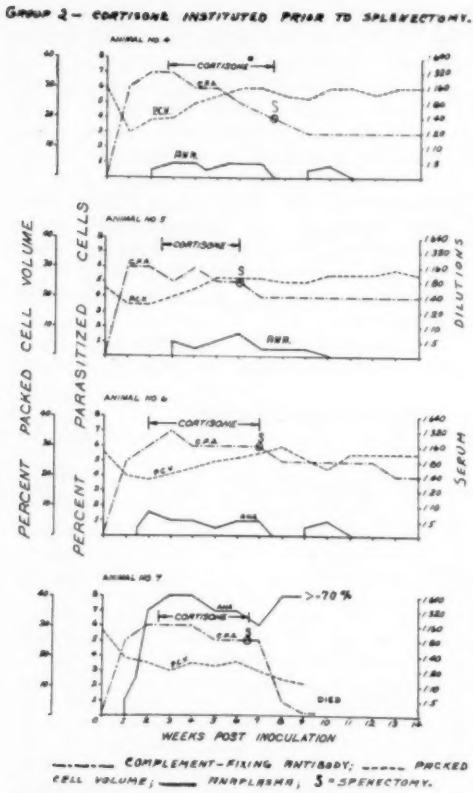


Fig. 1—Complement-fixing antibody and hematological responses of calves experimentally infected with Anaplasma. Infections were induced by intravenous injections of 50 ml. of heparinized bovine blood with 70 per cent Anaplasma-infected erythrocytes.



* Cortisone instituted intramuscularly, 1 mg. per pound of body weight, daily.

Fig. 2—Complement-fixing antibody and hematological responses of calves experimentally infected with Anaplasma. Infections were induced by intravenous injections of 50 ml. of heparinized bovine blood with 70 per cent Anaplasma-infected erythrocytes.

cytes, with a maximum number of parasitized cells at the end of the second week (at which time animals 1 and 3 died); a slight increase of hematocrit level, followed by a sharp drop during the second week; and the complement-fixing antibody titer receded and was not demonstrable two weeks after splenectomy in a dilution of 1:5. All 3 animals had severe anemia after splenectomy; the erythrocyte counts and hemoglobin levels were reduced approximately 50 per cent. The average decreased lymphocyte and increased granulocyte counts were observed during the three-week period following splenectomy. In no instance were reticulocytes observed; however, polychromatic and stippled cells were seen at the time of erythrocytic crises. The icteric index did not increase in any of the experimental animals.

Group 2.—Cortisone was administered to the 4 animals in this group for varied periods prior to splenectomy. The results of serological and hematological tests for all 4 animals were similar to those of group 1 during the presplenectomy period with the exception of calf 7, which had an average of 7 per cent infected erythrocytes. No significant complement-fixing titer fluctuations were observed during the period of cortisone administration with the exception of calf 4, in which there was a decrease of complement-fixing titer from 1:320 before cortisone treatment, to 1:40 at the end of the cortisone treatment. After splenectomy, calf 7 relapsed and followed the hematological and serological pattern of animals in group 1.

The immunological states of the other 3 animals were not significantly altered. Anaplasma was occasionally found in blood smears, but never exceeded the percentage of infection before splenectomy. A slight drop of the hematocrit level was observed during the second week postsplenectomy, followed by prompt regeneration. A decrease in complement-fixing titer of not more than one serial dilution was observed during the five-week period following splenectomy. With the exception of calf 7, which developed anemia and relapsed, the other animals had only slight anemia. Few reticulocytes, and a number of polychromatic and stippled cells, were found in these animals. The average presplenectomy lymphocyte percentage was maintained postsplenectomy.

Group 3.—The three calves in this group received cortisone for varied periods before and after splenectomy. Results resembling those for calves in group 2 were obtained. Protection against relapse was apparent in all animals. After splenectomy, the erythrocyte counts and hemoglobin values first fell slightly, then showed a slight but constant increase. Complement-fixing titers remained unaffected for at least three weeks after splenectomy.

Group 4.—The 3 calves in this group received cortisone on the day of splenectomy and for varied periods thereafter. Severe anemia, with a heavy recrudescence of the organism in the blood, was observed. Two of the animals succumbed to the infection and 1 gradually recovered following postsplenectomy relapse. Complement-

fixing antibody was not observed in serum dilutions of 1:5, two weeks after splenectomy.

Group 5.—The 6 calves in this group served as Anaplasma-free controls and were treated in the manner outlined (table 1).

DISCUSSION

Observation of the effect of splenectomy in precipitating relapse of the dormant form of anaplasmosis has substantiated the opinion that the spleen occupies a central defense position in this disease. Administration of adrenal steroid to Anaplasma carriers has resulted in paradoxical results of infection, depending on the presence or absence of the spleen. When cortisone was administered before splenectomy, over relatively long periods (with one exception), carriers showed no increase of Anaplasma and the immunological states were not significantly affected by splenectomy. However, when cortisone was administered at the time of splenectomy and for varied periods thereafter, progress of the infection became aggravated and resembled the usual postsplenectomy relapses.

Though more work will be needed to further evaluate the preliminary impression, the findings indicate a causal relationship between steroid therapy and the function of the spleen in bringing about and maintaining immunity to anaplasmosis. The fact that animals to which corti-

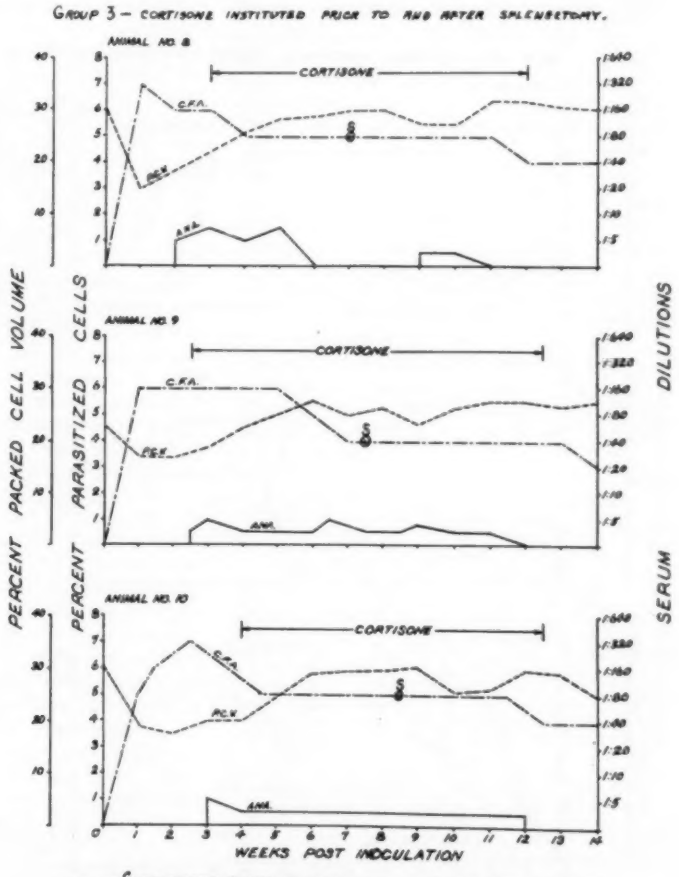


Fig. 3—Complement-fixing antibody and hematological responses of calves experimentally infected with Anaplasma. Infections were induced by intravenous injections of 50 ml. of heparinized bovine blood with 70 per cent Anaplasma-infected erythrocytes.

Cortisone instituted intramuscularly, 1 mg. per pound of body weight, daily.

sone was administered before splenectomy developed only slight or no hypertrophy of the spleen (as shown in the succeeding paper), suggests that cortisone interferes with splenic tissue in such a way as to reflect a decrease of its compensatory and destructive capacity for the organism. An exception was calf 7, which relapsed after splenectomy.

Possible explanations may be the failure of individual animals to respond to cortisone treatment, or the more aggravated condition of this animal, indicated by an increased number of infected erythrocytes at the time the cortisone treatment was started. It appears that cortisone did not function properly to induce postsplenectomy protection, whereas, in animals which were protected, cortisone might have stimulated extrasplenic tissue (in particular, bone marrow), to sufficient anti-Anaplasma activity to compensate for the removal of the spleen. An excessive stimulation of the bone marrow by cortisone was indicated by an increase of circulating reticulocytes and polychromatic and stippled cells. According to Gradwohl,[4] Britton obtained evidence that polychromasia and stippling are identical with reticulation. Adequacy of the bone marrow of cortisone-treated animals is further judged by the increased erythrocyte counts and hemoglobin levels. Similar findings regarding the influence of adrenal steroid on the rate of erythrocyte production have been reported in the various types of hemolytic disease of man.[1, 2, 5, 7]

Clinical signs of the disease were associ-

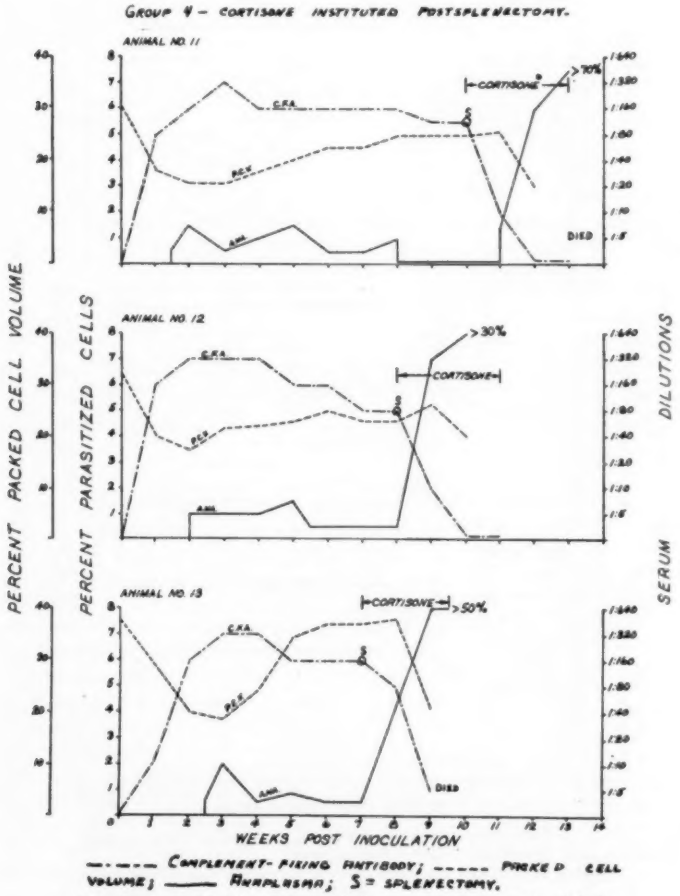


Fig. 4—Complement-fixing antibody and hematological responses of calves experimentally infected with Anaplasma. Infections were induced by intravenous injections of 50 ml. of heparinized bovine blood with 70 per cent Anaplasma-infected erythrocytes.

* Cortisone instituted intramuscularly, 1 mg. per pound of body weight, daily.

ated with severe anemia, produced by the initial parasitic attack approximately two weeks after infection; such signs gradually receded during the time the hematocrit level showed progressive improvement. In contrast to the severe icterus manifested in the natural cases in adult cattle, there was no similar indication in the animals studied. While infected control animals developed relative lymphocytopenia and granulocytosis postsplenectomy, the inflammatory cell picture did not change for the carriers to which cortisone was administered prior to splenectomy. The adrenal cortex is considered one of the important factors controlling the release of lymphocytes into the peripheral blood stream. Failure of cortisone to suppress the lymphocytic mobilization may have resulted from the relatively low cortisone dosage or from the stimulating effect of cortisone on bone marrow, indicated by the ability of this tissue to maintain effectively the cellular equilibrium of the blood. The latter possibility is substantiated by the generally accepted opinion that, under abnormal stimuli, the lymphocytes, which are regularly produced in lymphatic tissue, and the hemocytoblasts, which are regularly produced in myeloid tissue, are able to develop into any cells of the blood and connective tissues.[6]

Disappearance of complement-fixing antibody from the blood of splenectomized carriers indicates that the spleen has a distinct role in producing and maintaining such antibody. Disappearance of complement-fixing antibody in splenectomized carrier calves was concomitant with Anaplasma invasion of the erythrocytes. Therefore, it would appear that the amount of complement-fixing antibody present after splenectomy may materially influence the form and ultimate outcome of the disease. Due to absence of the spleen, no further antibody is produced and, consequently, the animal will again become susceptible. However, postsplenectomy persistence of complement-fixing antibody, although at a slightly decreased level, was observed in animals to which cortisone was administered prior to splenectomy. This indicates that, in cortisone-treated animals, the spleen does not function as the sole organ in setting up immunity and inducing the formation of complement-fixing antibody. The relationship between the postsplenectomy persistence of complement-fixing antibody and the failure of such animals to relapse indicated the need for further studies to evaluate the protective nature, if any, of complement-fixing antibody. Such studies are now in progress.

It is reasonable to assume, on the basis of data presented, that extrasplenic anti-Anaplasma activity accounts for the postsplenectomy immunity. It is now understandable why, after splenectomy, the initial period of parasitic multiplication is followed by a return to latency.

SUMMARY

The relapse, which ordinarily follows splenectomy in Anaplasma-infected calves, did not occur in 6 of 7 calves which had been treated for four to five weeks before splenectomy with adrenal cortex steroid. Moreover, in these animals, the titers of complement-fixing antibody did not fall significantly after splenectomy. Infected calves, which received this hormone at splenectomy and for varied periods thereafter, showed a similar postsplenectomy increase in Anaplasma and decrease in complement-fixing antibody, as observed in nontreated infected animals. A more proficient bone marrow activity, indicated by increased blood cell counts and an increase in circulating reticulocytes, was manifested in animals to which cortisone was administered for varied periods prior to splenectomy.

Further observations are necessary to evaluate the importance and influence of adrenal cortex hormone on Anaplasma immunity.

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Effect of Cortisone on the Mechanism of Anaplasma Immunity in Experimentally Infected Calves
II. Studies of Pathological Changes

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THIS REPORT DESCRIBES the gross and microscopic abnormalities observed in the tissues of calves experimentally infected with *Anaplasma marginale*. The immunological aspects of these calves were presented in the preceding report.[1] The 4 infected calves of group 2 received cortisone for periods of four to five weeks prior to splenectomy, while the 3 in group 3 received the hormone over periods of four to five weeks prior to and after splenectomy. The relapse which ordinarily follows splenectomy in Anaplasma-infected calves did not occur in 6 of 7 calves in the two groups. The 3 calves of group 1, which served as infected controls and the 3 calves of group 4, which were experimentally infected and received cortisone on the day of splenectomy and for approximately three weeks thereafter, demonstrated severe anemia, with a heavy recrudescence of the organism in the blood. The protocol regarding the cortisone treatment of experimentally infected calves and Anaplasma-free control animals is shown in table 1 of the preceding study.[1]

A study of the pathological changes was not contemplated at the beginning of the experiment. When the studies reported in the first paper revealed unexpected results, it seemed important to study the available tissues, to delineate, if possible, the mechanisms involved.

MATERIALS AND METHODS

The following tissues of infected and Anaplasma-free control animals were prepared for histological examination: (1) spleens obtained at the time of splenectomy; (2) livers, sternal bone marrows, hepatic lymph nodes, and kidneys obtained at necropsy; (3) adrenal glands of calf 2, which relapsed approximately two weeks post-splenectomy, obtained at necropsy.

The tissues were fixed in 10 per cent formaldehyde-saline solution (pH 6.3 to 6.5), transferred through a series of methyl alcohol solutions, embedded in paraffin, sectioned at approximately 5 μ, and stained with Harris' hematoxylin and eosin. Bone marrow sections were decalcified by immersion in 5 per cent nitric acid for six to eight hours, washed for 24 hours in running tap water, and treated as outlined above.

It is generally accepted that the spleen has a decisive role in the course of anaplasmosis. Therefore, it was decided that this organ should be investigated by determining its weight at splenectomy and establishing the ratios of spleen weight to body weight in the animals of groups 1 and 2.

RESULTS

GROSS PATHOLOGY

Calves which relapsed, or relapsed and died, had varying degrees of anemia of the mucous membranes, without indication of icterus. The lymph nodes, in general, were slightly enlarged and somewhat edematous; the spleens were enlarged approximately one and one half times, showing dark, protruded parenchymatous tissue on the cut surface. Comparative figures of the spleen : body weight ratios of animals in groups 1 and 2 are shown (table 1).

TABLE 1—Effect of Cortisone on Spleen : Body Weight Ratios in Calves Experimentally Infected with Anaplasma

Treatment	Animal (No.)	Weight		Post-splenectomy results
		Spleen (Gm.)	Body (lb.)	
Group 1, no cortisone	1	1,472	206	Relapse; died.
	2	1,102	199	Relapse
	3	1,173	192	Relapse; died.
Group 2, cortisone before splenectomy	4	893	189	No relapse
	5	881	201	No relapse
	6	976	193	No relapse
	7	1,237	201	Relapse

The splenic weights of calves in group 2 were lower than those of group 1, with the exception of calf 7, which had a spleen comparable to those of calves in group 1. No notable liver changes were detected.

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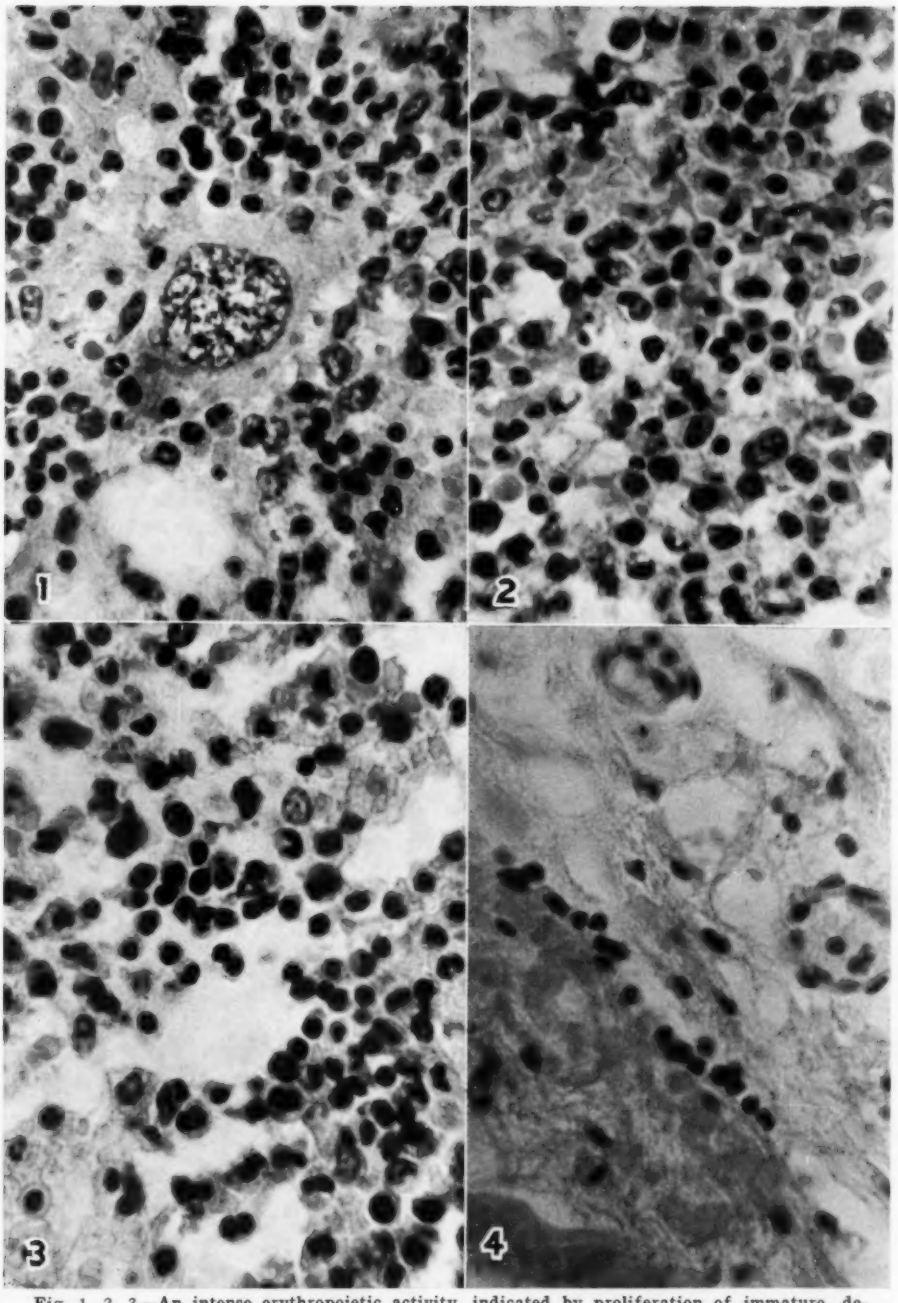
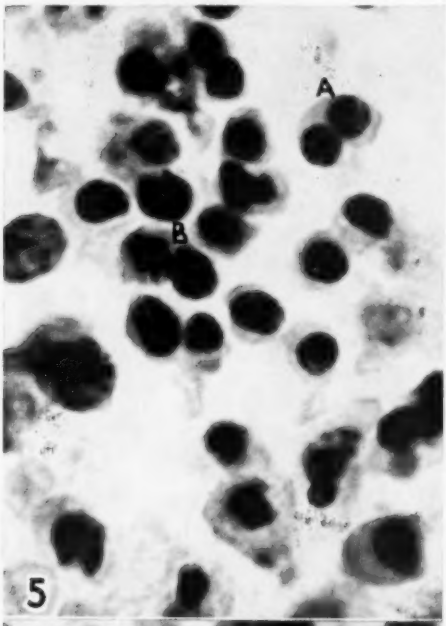
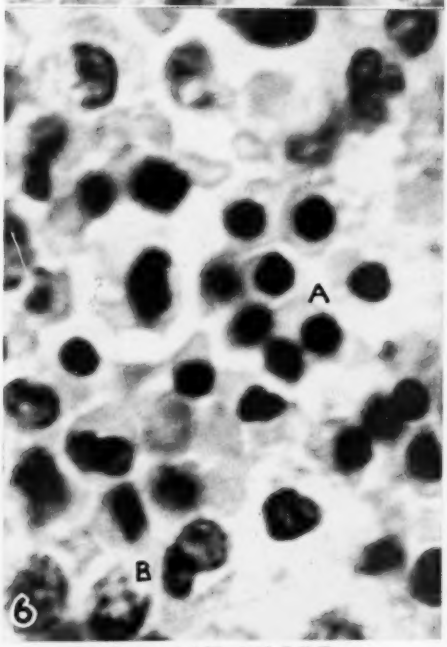


Fig. 1, 2, 3—An intense erythropoietic activity, indicated by proliferation of immature, developmental erythrocytic forms in bone marrow of calves 4, 8, and 9, respectively. Calf 4 received cortisone before splenectomy; calves 8 and 9 before and after splenectomy. x 900.

Fig. 4—Depression of erythropoietic activity in the bone marrow of calf 1, which was infected and not treated with cortisone and relapsed postsplenectomy. x 900.





Developmental Myeloid Cell Forms

Fig. 5—(A and B) Erythrocytic precursors in mitosis. x 1200.

Fig. 6—(A) Group of normoblasts; (B) Reticular cells containing granular material. x 1200.

The mucous membranes of the gallbladders were slightly thickened and inflamed. The bone marrow appeared intensely purple. Petechiae were occasionally observed on the pericardium, pleura, and diaphragm. The animals which did not relapse had no visible anatomical changes.

HISTOPATHOLOGY

Bone Marrow.—All infected animals treated with cortisone exhibited an intense erythropoiesis, indicated by excessive proliferation of immature, developmental erythrocytic forms (fig. 1–3), in contrast to animals not treated with cortisone, which relapsed following splenectomy and had depressed erythropoietic activity (fig. 4). Erythroblasts and normoblasts were most numerous in the marrow of animals which had intense erythropoiesis. Many of these cells were in various stages of mitosis (fig. 5, 6).

With the exception of several cell forms with granular cytoplasm, thought to be immature eosinophils, the myelocytes were

Fig. 7—Moderate lymphoid exhaustion of the nodule of the hepatic lymph node of calf 3. x 120.

infrequent. The reticular cells composing the stroma of treated and nontreated infected animals appeared in various stages of mitotic proliferation, containing granular and amorphous material in the cytoplasms (fig. 5, 6). The megakaryocytes were apparently normal in number and form. Unidentified bluish pink material, suggesting dissolved hemoglobin, was observed in large areas of the sections, particularly in tissues of animals which relapsed following splenectomy.

Spleen.—Various degrees of exhaustion of the germinal centers were indicated by a decrease of lymphoblasts and an increase of vacuoles and degenerated reticular cells of these areas (fig. 8, 9). In animals that relapsed and died, the diameter of the lymphocytic ring zone of the white pulp was reduced to one third of that of the similar area of the control animal. However, "mesenchymal activation" was indicated by increased numbers of lymphocytic forms within the red pulp. The red pulp portion of the venous sinuses and the Billroth cords appeared enlarged and contained many reticular cells and various sizes of mononuclear cells which, with the increased numbers of lymphocytes, gave the red pulp an intense cellular appearance. Many sections contained hemosiderin-like pigment.

Liver.—The most prominent change in animals which relapsed postsplenectomy occurred in the capsule of Glisson, which was infiltrated with leukocytes (fig. 11). The portal veins of this area were dilated and the endothelial cells were swollen. The number of undifferentiated perivascular cells increased, which made the portal vein prominent. The sinusoids were dilated and contained neutrophils, lymphocytes, nucleated erythrocytes, and swollen Kupffer cells. The blood in the vessels contained many leukocytes and various nucleated erythrocyte forms. The space of Disse was visible in many of the sinusoids. Foci of erythroblasts and normoblasts, in various developmental stages, were found in the sinusoids of calves 6 and 9, suggesting hematopoiesis (fig. 10).

Slight albuminous degeneration of the cytoplasm of hepatic cells was observed in at least 1 animal from each infected group. Tissues of calves 2 and 11 showed lobular degeneration, central in origin and successively involving more of the lobule; there were many round vacuoles in these areas, suggesting fatty degeneration. Numerous areas of focal necrosis, some of which were in the process of encapsulation by connective tissues, were observed in the tissue of calf 7.

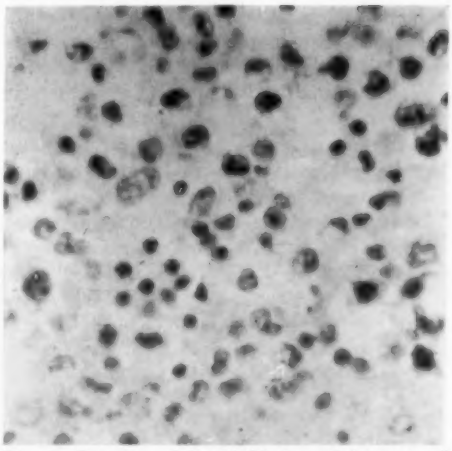


Fig. 8—Decrease of lymphoblasts and increase of vacuoles and degenerated reticular cells in the splenic germinal center of calf 1. x 900.

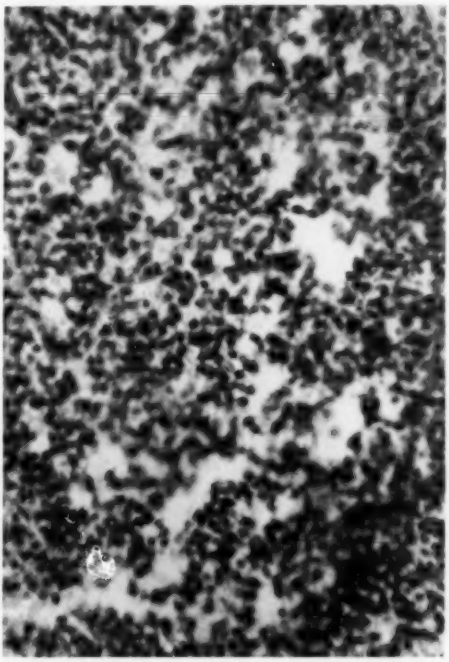


Fig. 9—Exhaustion and edema of the lymphocyte germinal center in spleen of calf 7. x 640.

Hepatic Lymph Node.—Moderate lymphoid exhaustion of the nodules (fig. 7) was observed in both treated and nontreated infected animals. Edema and lymphocytic infiltration of the capsule were seen in sections from calves 2, 3, and 7. In all cases, the cortical areas contained many reticular macrophages with clear cytoplasm. Edema of the hilus area, with accumulation of lymphocytes and mononuclear cells, was found in calves 1, 7, and 13.

Kidney.—Lesions of the kidney were found only in animals of groups 1 (infected, no cortisone) and 4 (infected, cortisone administered postsplenectomy). Many of the glomeruli were excessively cellular and some had masses of closely packed, large nuclei. The glomerular epithelial lining was swollen, overfilling the glomerules and obliterating Bowman's space. Some of the interstitial connective tissue contained hyperchromatic nuclei. Tubular lumina contained erythrocytes, desquamated epithelium, and amorphous material.

Adrenals.—The cytoplasm of the cells of the zona fasciculata contained no vacuoles or lipoid droplets, indicating exhaustion (fig. 12). Nuclei of cells at the juncture of the zona glomerulosa and zona fasciculata were numerous, giving this area an unusually cellular appearance.

DISCUSSION

It is apparent, from the data presented, that Anaplasma infection induces both cellular and humoral mechanisms of defense. A distinguishing characteristic of Anaplasma infection is that the agent is constantly present within the blood vascular system, as indicated by transmission experiments. Therefore, it is assumed that the cellular defense exists primarily in the organs possessing blood sinuses in addition to the ordinary capillaries. In these areas, the rate of the blood flow is decreased and

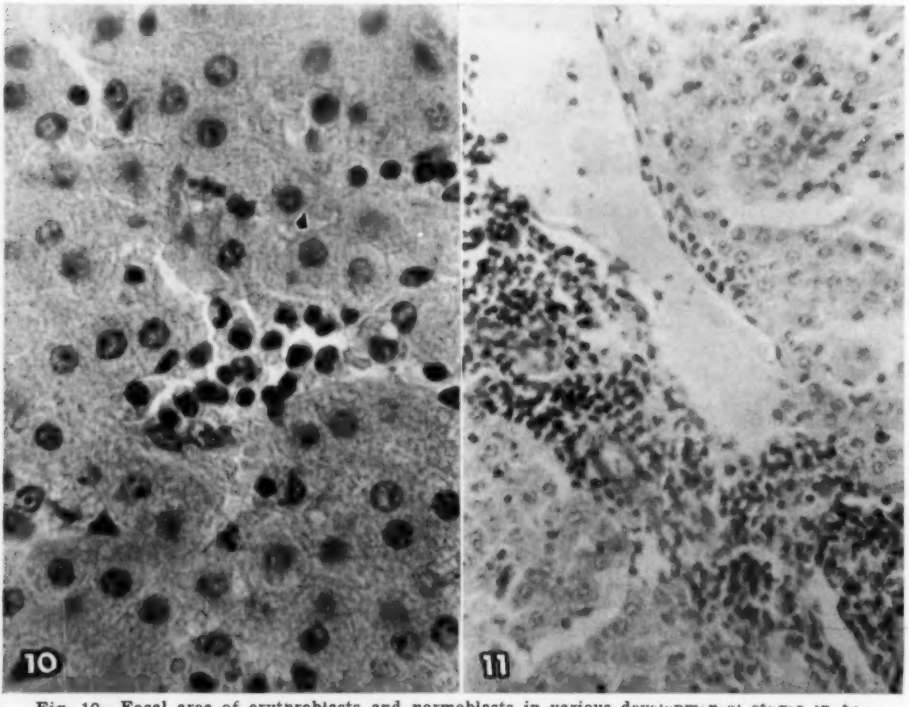


Fig. 10—Focal area of erythroblasts and normoblasts in various developmental stages in the liver tissue of calf 6. x 900.

Fig. 11—Inflammatory cell reaction in the capsule of Glisson of calf 7. This was the most prominent hepatic change in calves which relapsed postsplenectomy. x 640.

there is a maximum exposure time of infected erythrocytes to the potentially phagocytic cells which line the sinuses and the loose tissues around them. Selective localization of the organism in these areas is indicated by hypertrophy of the spleen and hyperplasia of erythropoietic components of the bone marrow. Clinical severity of the infection could depend upon the parasitizing potential of the infectious agent, which may be determined primarily by the amount of the infectious dose. However, variations in virulence have been noticed, and the ability of certain individual hosts to convert a general, progressive type of infection into a localized, regressive type has been observed.[2]

Successful resistance, as observed in carriers which failed to relapse postsplenectomy, is indicated by active proliferation and hypertrophy of macrophages of the splenic cords, the Kupffer cells of the liver, and the reticular cells of the bone marrow. The spleens of carriers which succumbed postsplenectomy revealed dysfunction or exhaustion of the germinal lymphocyte centers. Since these animals showed an extreme compensatory hypertrophy of the spleen as a whole, the term "exhaustion" seems appropriate. The splenic abnormalities agree with the general assumption that the spleen is of paramount importance in developing and maintaining immunity to anaplasmosis.

The intensity of the erythropoietic response was most pronounced in myeloid tissues of animals to which cortisone was administered prior to splenectomy. Conversely, none of the myeloid tissues revealed intensive granulocyte production, which may indicate an inferior defense role of circulating granulocytes, as compared with fixed or free phagocytes of the reticuloendothelial system. Foci of erythropoietic activity were observed in liver sections of 2 animals which were given cortisone. The ability of the liver to assume its embryonic activity may have a favorable effect on the ultimate outcome of the infection. Thus, the compensatory interaction between the spleen, bone marrow, and other tissues capable of setting up an erythropoietic activity appears significant. Removal of the spleen, when bone marrow is depressed due to Anaplasma infection, and when there is a lack of extramyeloid hematopoietic activity, would disturb the existing balance of defense forces and result in anemia.

As measured by clinical appearance and hematocrit levels, the crisis of the disease occurred approximately two weeks after infection.[1] The complement-fixing antibody was detectable in approximately its highest concentration at seven days postinfection. Following splenectomy, the complement-fixing antibody persisted, although at a slightly reduced level, in animals which failed to relapse. The appearance and persistence of this antibody shortly before or at the time of probable activity of the reticuloendothelial system, may indicate that the antibody acts in conjunction with cellular phagocytes in suppressing the multiplication of Anaplasma. It is likely that the complement-fixing antibody is capable of sensitizing the parasite and rendering it more susceptible to phagocytosis. Under these circumstances, increased phagocytic activity may depend upon activity of an opsonic antibody for establishment and maintenance of anaplasmosis immunity.

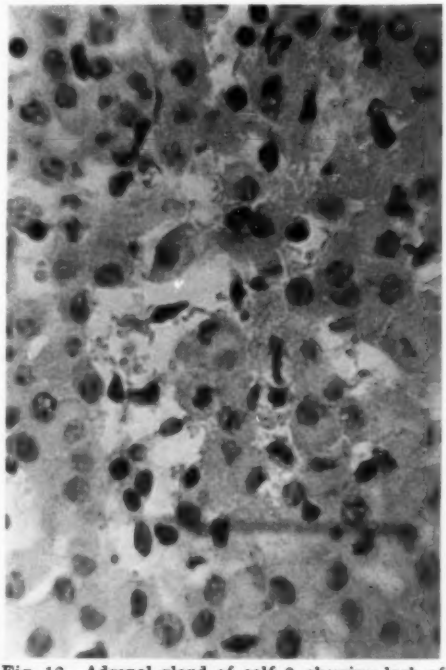


Fig. 12—Adrenal gland of calf 2, showing lack of vacuolization of cells.

Adrenal abnormalities could not be evaluated on the basis of microscopic appearance of tissues of a single animal. Therefore, significance is not attributed to the possibility that protection may have resulted from parenterally-administered cortical steroid. Experiments designed to investigate such a possibility are now in progress. It is known that the adrenal cortex responds in a nonspecific manner to a wide variety of stresses. Cortisone could have contributed to lessening the stress factors consequent to splenectomy, although it is less probable that postsplenectomy protection has resulted mainly from elimination of the stress effect.

Under the conditions of these experiments, it appears that the Anaplasma agent is responsible for initiating a series of defense reactions. These reactions are characterized primarily by active engagement of lymphatic and myeloid tissues capable of producing humoral and cellular forces, the latter being either compensatory or defensive in nature.

SUMMARY

Hypertrophy of the spleen, inflammation and swelling of the mucous membrane of the gallbladder, purplish discoloration of the bone marrow, and petechiae of the pericardium, pleura, and diaphragm were the most characteristic gross abnormalities observed in calves infected with Anaplasma, which relapsed, or relapsed and died, postsplenectomy.

Animals which relapsed after splenectomy had depressed erythropoietic activity of the bone marrow, in contrast to an intense bone marrow activity observed in animals to which cortisone had been administered prior to splenectomy. Leukocytic infiltration of the capsule of Glisson was the most pronounced hepatic change in animals which relapsed postsplenectomy. Hematopoietic activity of the liver was indicated by the presence of foci of erythroblasts and normoblasts in 2 cortisone-treated infected animals.

General reduction of white pulp of the malpighian follicles, with a decreased number of lymphoblasts and an increase of vacuoles and degenerated reticular cells of the germinal centers, was more pronounced in spleens of animals which did not receive cortisone. The red pulp portions of the venous sinuses and the Billroth cords of the spleen were generally enlarged and contained many reticular cells of various sizes, giving the red pulp a cellular appearance.

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Studies of the Bovine Adrenal Gland. II. The Histological and Cytochemical Effects of the Administration of 1, 1 Dichloro-2, 2-Bis (P-Chlorophenyl) Ethane on the Adrenal Cortices of Dairy Calves

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THE ORAL ADMINISTRATION of the chlorinated hydrocarbon compound 1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethane (DDD) produces a selective atrophy of the inner zones of the canine adrenal cortex, while the zona glomerulosa shows little change.[11-14] The destruction of the inner cortical zones, as observed on histological examination, has been correlated with a fall in plasma and urinary 17-hydroxycorticosteroids.[3, 4] These facts lend further evidence to the glucocorticoid-producing nature of the zona fasciculata.

When fed to rats and rabbits, DDD causes little or no alteration in the histological structure of the adrenal cortices,[8, 10] but does cause some degree of cortical dysfunction, as shown by increased sensitivity to insulin, decrease in eosinophil response to adrenocorticotrophic hormone (ACTH) injections, and decrease in the stress response to a cold environment.[2] Sheehan *et al.*[15] were unable to alter the signs of adrenal cortical hyperactivity in a case of Cushing's disease by giving DDD, demonstrating the insensitivity of the human adrenal cortex to the cytopathogenic effect of this compound. It appears, therefore, that there is considerable variation among species as to the effect of DDD administration on the adrenals.

There are no reports on the effects of DDD on the adrenal cortices of domestic ruminants. Thus, the following investigation was undertaken to study the histological and cytochemical changes, if any, which might occur in the adrenal cortices of dairy calves when this compound is given.

MATERIALS AND METHODS

Animals and Treatment.—A total of 11 Holstein-Friesian heifer calves were used in these experiments. They were maintained in small pens and fed milk plus a nutritionally adequate dairy calf ration of ground feed and good quality alfalfa hay. 1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethane (Rhothane *) was administered *per os*, in gelatin capsules, once daily at a rate of 50 mg. per kilogram of body weight.

In a preliminary experiment (table 1), 3 calves, which initially averaged 118 kg. in body weight, were given DDD for 7.5 to 12.0 weeks. Since histological observations of the adrenal glands from these calves indicated that marked changes had occurred, and that administration of the compound over a much shorter time might also produce similar changes, 5 calves, weighing between 38 and 58 kg., were given DDD for three, six, nine, 12, and 15 days, respectively. Results of these trials (table 1) showed that marked adrenal changes occurred by the ninth day after the beginning of treatment. To verify the previous findings, 3 additional calves of similar size were given DDD for ten days. Only 1 control animal was used in these experiments, since data are available on numerous calf adrenals from previous studies in this laboratory.[16, 17]

Technique Procedures.—All animals were weighed weekly and at the time of slaughter. Total and differential blood counts were made weekly on calves which were given DDD for 7.5 to 12.0 weeks.

Tissues collected for study included sections from the pancreas, liver, and kidneys, and from the pituitary, thyroid, and adrenal glands. Adrenal and pituitary gland weights were obtained when the animals were killed. Only the observations on the adrenal glands will be presented in this paper; the other organs are under study and the results will be reported.

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Techniques for preparing the tissue included those for paraffin-embedded and fixed, frozen tissues. These techniques, as well as those used for studying birefringent material, tissue cholesterol, and estimations of lipid content, have been previously described.[17, 18] Cytochemical tests for the presence of adrenal ketosteroids were not employed, since recent evidence[9] indicates that these tests are of questionable value.

RESULTS

All calves remained in apparent good health throughout the experiments. Rates of weight gain were not determined for animals on the short-term experiments. The 3 animals which were given DDD for 7.5 to 12.0 weeks gained at an average rate of 5 kg. per week. This figure is approximately average for control calves of similar weight which were used in other experiments in our laboratories.[16] The appetites of all 3 calves gradually increased, until the daily feed intake of each was approximately twice that of untreated calves, and it appeared that they would have consumed additional feed had it been offered. After approximately the sixth week of the experiment, the left abdominal walls of the 3 calves were observed to protrude abnormally. At necropsy, it was found that the rumen of each was considerably enlarged and contained a greater amount of ingesta than usual. The ingesta also had a less fermented appearance than was commonly observed. The reticulum and omasum of each animal appeared to be normal in size and content. The feces were of normal consistency throughout the experiment.

Total and differential blood counts were made weekly on the calves which were given DDD for 7.5 to 12.0 weeks. Significant deviations from the normal[6, 7] were not observed in these values. The eosinophil percentages remained under 3 per cent in all animals. The total weights (table 1) of the adrenal glands of all treated animals were well within normal ranges[16] for their individual body weights.

The histological and cytological results are reported according to the length of time over which the compound was administered, rather than the chronological order of the experiments (table 1).

Oil Red O-Stainable Lipids.—As indicated in an earlier report,[17] in the adrenal glands of untreated, clinically normal calves there is rarely any histological or cytochemical evidence of lipid droplets. The adrenal cortex of the control animal was also essentially negative for lipid material in droplet form (table 1).

Three days after the beginning of DDD administration, moderate numbers of lipid droplets were present in the cells of the zona fasciculata and the zona glomerulosa. A small proportion of cells (*see* under "Cytological Studies") were greatly hypertrophied (fig. 2) and contained large numbers of coarse oil red O-stainable and cholesterol-positive inclusions. In the adrenal glands of the calf which was given DDD for six days, the quantity of lipid

TABLE 1—Body and Total Adrenal Weights, and Cytological Estimations of Oil Red O-Stainable Lipids, Cholesterol, and Birefringent Crystals in the Adrenal Cortices of Dairy Calves Which Were Fed DDD

No. of animals	Treatment	Terminal body wt. (kg.)	Total adrenal wt. (Gm.)	Microscopic determination	Zona glomerulosa	Zona fasciculata–zona reticularis
1	Control	42	3.5	Oil Red O	+6	—
				Cholesterol	—	—
				Birefringence	—	—
1	DDD,* 3 days	38	3.9	Oil Red O	+++	+++
				Cholesterol	+	—
				Birefringence	++	+
1	DDD, 6 days	47	4.4	Oil Red O	—	++++
				Cholesterol	—	++++
				Birefringence	—	+
6	DDD, 9 to 15 days †	47, av.	3.5, av.	Oil Red O	+	++++
				Cholesterol	++	+++++
				Birefringence	++	+++++
3	DDD, 7½ to 12 weeks §	162, av. (118, av.)‖	6.3, av.	Oil Red O	—	++ ‡
				Cholesterol	—	—
				Birefringence	—	—

The plus and minus signs are based on visual inspection, microscopically, and are to be interpreted as follows: — indicates absence of the substance in question; +++++ indicates that a cell or area was heavily loaded with the substance in question.

* Given *per os* once daily in a gelatin capsule at a dosage of 50 mg./kg. body weight. † One animal for nine days; 3 for ten days; 1 for 12 days; 1 for 15 days. ‡ Oil Red O Stain was localized in the zona reticularis. § Two animals for 7.5 weeks; 1 for 12.0 weeks. ‖ Initial body weight.

droplet material in the zona fasciculata was increased, while none was present in the zona glomerulosa. In the adrenal glands of this animal, as well as in those of animals which were given the compound for nine, ten, 12, and 15 days, the lipid material could be seen grossly as a band of chalky white material which occupied from one half to two thirds of the zona fasciculata and parts of the zona reticularis.

Histological examination of the adrenal cortices of animals which were treated for nine to 15 days, showed massive numbers of coarse cytoplasmic lipid droplets (up to 5 μ in diameter) in the affected parts of the zona fasciculata and the zona reticularis. The zona glomerulosa was only slightly involved.

Grossly, the adrenal glands of calves which were given DDD for 7.5 to 12.0 weeks appeared pale pink on cut section. Studies of fixed, frozen sections from the adrenals of these calves revealed that only small amounts of coarse lipid droplet material were present in cells of the zona reticularis, while the remainder of the cortex was free of stainable lipids.

Qualitative Cholesterol Studies.—The histochemical test for the presence of tissue cholesterol was strongly positive in the fascicular zones of adrenals from all calves which were given DDD for six to 15 days, while only slight reactions were seen in the glomerular zones (table 1). The adrenal cortex of the control animal was negative for cholesterol, which is typical for domestic ruminants. In calves which were treated for 7.5 to 12.0 weeks, all zones were negative. Thus, the pattern of cholesterol accumulation was observed to follow the pattern of lipid appearance.

Birefringent Crystals.—Examination of sections under polarized light showed heavy concentrations of extracellular acicular birefringent crystals (fig. 1) in the zona fasciculata of the adrenals from animals which were treated for nine to 15 days. These structures, which were of similar size to those previously reported,[17] were thought to be precipitation artifacts of intracellular lipid substances which were present in unusually large amounts. No crystals were observed in the adrenal glands of calves which were treated for 7.5 to 12.0 weeks. Thus, the presence or absence of these crystals, like the cholesterol accumulation, closely followed the pattern of lipid appearance.

Fig. 1—Extracellular acicular birefringent crystals in the zona fasciculata of the adrenal gland of a calf which was given DDD for ten days. x 1,100.

Cytological Studies of Paraffin Sections.—In the adrenals of the calf which was given DDD for three days, a small proportion of isolated cells of the inner portion of the zona fasciculata were greatly hypertrophied (fig. 2). These cells measured up to 35 μ in diameter and were characterized

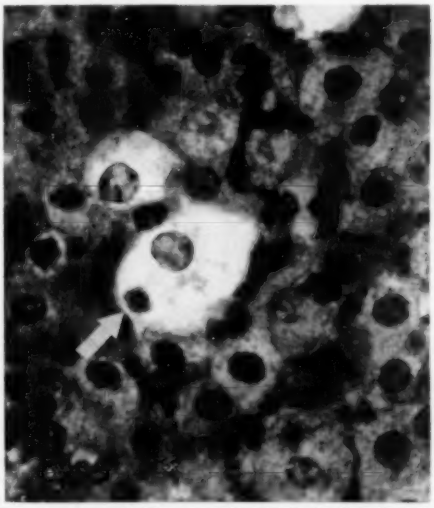

Fig. 2—A markedly hypertrophied, lipid-laden cell (arrow) in the zona fasciculata of the adrenal gland of a calf which was given DDD for three days. Note the spherical fuchsinophilic cytoplasmic body at tip of pointer. x 1,000.

by the presence of almost totally disintegrated cytoplasm. A peculiar finding, of unknown significance, was the presence of a spherical, fuchsinophilic body (3 to 4 μ in diameter) in nearly all of the markedly hypertrophied cells. The nuclei stained less densely, were slightly enlarged, and varied from turgid to slightly flaccid in appearance. A majority of the cells of both the fascicular and glomerular zones contained small cytoplasmic vacuoles which measured up to 3 μ in diameter. In the adrenal zona fasciculata of the calf which was treated for six days, the majority of parenchymal cells were hypertrophied to sizes varying between 15 to 20 μ in diameter, as compared with those of the control animal, which were approximately 10 to 12 μ in diameter. The cytoplasm varied from markedly vacuolated to almost totally disintegrated in appearance (fig. 3). The majority of the cells contained nuclei which were round and normal in appearance but which, in many cells, appeared to be moderately shrunken. A small proportion of cells had a markedly shrunken and degenerated cytoplasm with pyknotic nuclei.

Fig. 3—Cells of the zona fasciculata of the adrenal gland of a calf which was given DDD for six days. Note cells in various stages of hypertrophy and degeneration. The arrow points to a lipid-poor cell which is characteristic of the normal bovine adrenal zona fasciculata. The cell in the center of the photograph shows a pyknotic nucleus, and is one which contained large aggregates of oil red O-stainable and cholesterol-positive inclusions. x 1,150.

Petechial and ecchymotic hemorrhages were observed primarily in the glomerular and fascicular zones of the adrenal gland from the calf which was given DDD for nine days. Other than small areas of hyperemic sinusoids, major vascular abnormalities were not observed in any other animal in these experiments unless otherwise specified.

Examination of the adrenal cortices of those calves which were given DDD over a period of nine to 15 days showed a massive cellular hypertrophy and degeneration in the inner portions of the fascicular zones (fig. 4), characterized by the presence of cells (15 to 30 μ in diameter) containing a highly vacuolated to a nearly completely disintegrated cytoplasm. The nuclei of these cells were markedly shrunken, with folded nuclear membranes, and contained disorganized and usually darkly staining chromatin material. In some instances, the inner one half to two thirds of the zona fasciculata were involved, while the zona reticularis was relatively free of cytological changes. In other instances, the zona fasciculata was involved as described and, in addition, the zona reticularis was largely replaced by collagenous connective tissue. Mitotic activity was uniformly low in all zones of the adrenal cortices from all calves which were given DDD for three to 15 days.

Studies of paraffin sections of glands from the 3 animals which were given DDD for 7.5 to 12.0 weeks showed striking histological alterations in the architecture of the mid-fascicular zone, while the zona glomerulosa, the remainder of the zona fasciculata, and the zona reticularis appeared to be normal.

Major portions of the mid-fascicular zones in adrenals from these 3 animals were replaced by bands of collagenous connective tissue (100 to 300 μ in width), which resembled the adrenal capsule in structure (fig. 5). The widths of these bands of tissue appeared to be less than that of the involved parenchyma in animals which were given DDD over periods of 3 to 15 days. In addition, there was evidence of compression of the connective tissue band from the adjacent parenchyma. The cells of the zona fasciculata which were located peripherally to the connective

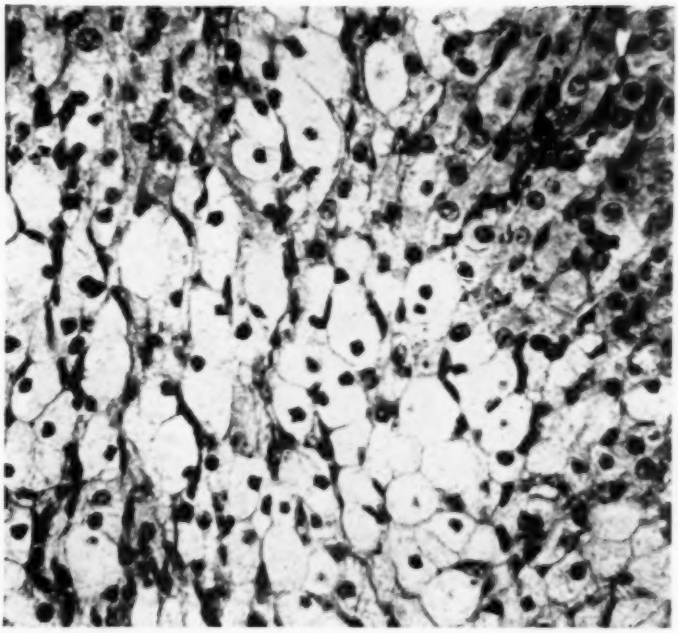

Fig. 4—Hypertrophied cells with pyknotic nuclei in the adrenal zona fasciculata of a calf which was given DDD for ten days. Note essentially normal cells in upper right corner of photograph. x 500.

tissue band showed no evidence of degenerative changes. The remaining portion of the zona fasciculata, located centrally to this band, was difficult to distinguish from the zona reticularis. Few cytoplasmic vacuoles were present, and only a few cells of this inner cortical area had shrunken nuclei with disorganized chromatin contents. Instead, the nuclei were either normal or increased in size and were turgid in appearance. Only an occasional mitotic figure was present.

DISCUSSION

The results of these experiments indicate that orally administered DDD was cytopathogenic for cells in the inner portions of the zona fasciculata and the zona reticularis. The outer fascicular zone and the entire glomerular zone were relatively free of these effects. The interval of treatment necessary to produce marked cytological changes in calves (6 to 15 days) is considerably less than the minimal durations of treatment which are reported for laboratory animals. The reason for this may be that lipid changes are more easily discernible in lipid-poor adrenal glands, such as those found in cattle.

It was observed in the adrenals of calves which were given DDD for 7.5 to 12.0 weeks that, except for a substantial band of connective tissue in the mid-fascicular zone, the adrenal cortices of these animals appeared relatively unaffected by the treatment. Stainable lipids, cholestrol, acicular birefringent crystals, and cells with a highly vacuolated cytoplasm were essentially absent from the cortex. These morphological findings suggested that, although the cytopathogenic effects on cells of the zona fasciculata and the zona reticularis were severe, they were also transient.

From external observations, it appeared that the adverse effects of this compound on the general physiological well-being of calves used in these experiments were negligible, in contrast to findings for dogs,[3,4] in which it produced weakness, tremor, anorexia, and death. The increased appetite, rumen distention, and presence of unfermented ingesta would seem to point to an inhibitory effect on the metabolic processes of the rumen microflora of the calves. While further supportive evidence is needed, it is speculated that sufficient adrenal cortical hormone production was present to meet minimal body needs.

Although the mechanism of action on the adrenal gland is unknown, it is specu-

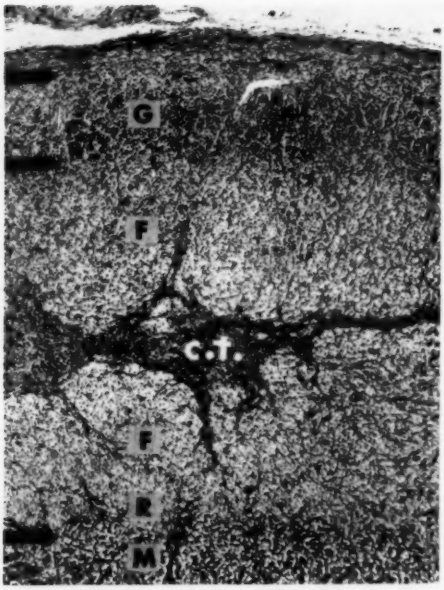

Fig. 5—Adrenal gland of a calf which was given DDD for seven and one half weeks. Note band of connective tissue (c.t.) in the mid-fascicular zone. Arrows point to boundaries of the various parenchymal zones. G = zona glomerulosa; F = zona fasciculata; R = zona reticularis; M = adrenal medulla. x 85.

lated [13] that DDD may block the action of pituitary ACTH on the adrenal cortex. It is also possible that it might prevent the formation or release of ACTH. The findings of Nichols and Gardner [13] and the results of others reviewed by these authors, favor the "ACTH block" theory, in that the zona glomerulosa has been observed to selectively escape the effects of DDD while the zona fasciculata and the zona reticularis, which are under ACTH influence, are extensively affected. In the experiments on calves reported here, it was found that the zona glomerulosa, the outer portions of the zona fasciculata, and varied portions of the zona reticularis were devoid of effects. These findings favor the "local effect" theory, although they do not militate strongly against the "ACTH block" theory, since regulation of the activities of each zone have not been unequivocally ascertained. To explain more exactly the authors' concept of the "local effect" theory, we suggest that the cytopathogenic effect of DDD in the involved areas may be dependent upon a slow rate of blood flow and the presence of sinusoidal vascular walls. The latter would, seemingly, be much more permeable than capillaries to substances in the blood stream since they are presumably incompletely lined by endothelium. It might be further postulated that the effect of DDD is also dependent upon the presence of a type of parenchymal cell which is relatively high in lipid content, since it has been shown [5] that this compound accumulates preferentially in body fats. It was reported [5] that DDD was present in a relatively high concentration in the adrenal gland. The "local effect" theory would help to explain why the inner adrenal cortical areas became refractive to the effects of DDD after the vascular pattern and, presumably, the vascular structure became markedly altered in these areas as the result of parenchymatous degeneration and interstitial tissue replacement.

It is difficult to assess the kind and degree of change in adrenal cortical cellular activity which attended the initial cellular hypertrophy and consequent degeneration in the glands of calves which were given DDD. One reason for this is that the cytological criteria of Deane [1] for relative adrenal cortical cellular activity apply primarily to glands which are normally lipid-rich and contain high levels of cholesterol. It seems reasonable to assume, however, that the first changes observed, cytoplasmic changes and nuclear hypertrophy with increased total lipids and cholesterol, are evidence of increased synthetic activity, or increased storage, or both. This may be due to a general stimulatory effect of DDD on adrenal cellular metabolic processes, to a failure to liberate steroids, or to a failure to convert precursors, such as cholesterol, to utilizable hormone. The presence of large accumulations of cholesterol-positive material would suggest that the latter is the case. The presence of large accumulations of acicular birefringent crystals is inexplicable in the presence of cellular hypertrophy. On the basis of previous results, obtained in studies of the adrenal zona glomerulosa,[17] these particles would indicate cellular inactivation. It is striking that the increase in total lipids and cholesterol causes the adrenal cortex of the calf to resemble the cytological picture seen in normal adrenal glands of laboratory animals and those of the horse.

Whatever the biochemical nature of the cytopathogenic effects of DDD on adrenal cortical cells might be, it is probable that they involve more than a fatal acceleration or deceleration of normal cellular processes. This assertion is made on the basis that, excluding the results obtained here, the fascicular and reticular zones have been found to be consistently low in cholesterol and acicular birefringent material,[1, 16] even where the adrenal cortices were extremely fatty.

SUMMARY AND CONCLUSIONS

Holstein-Friesian dairy calves (11) were used to study the effects on the adrenal glands of orally administered 1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethane (DDD). The compound was given in gelatin capsules at a dosage rate of 50 mg. per kilogram of body weight per day, for periods of three days to 12 weeks.

From external appearances, adverse physiological effects were not noticed in any of the animals during the course of treatment. In calves which were given DDD for 7.5 to 12.0 weeks, however, the daily feed intake was increased to the extent that it caused abnormal ruminal distention.

Cytological and cytochemical studies of the adrenal cortices of calves which were given DDD for three to 15 days indicated that this compound exerted a cytopathogenic effect on cells of the inner portions of the zona fasciculata and the zona reticularis, which eventually terminated in massive parenchymatous degeneration and interstitial tissue replacement in the affected areas. A prominent feature of this process was the accumulation of large amounts of oil red O-stainable material in coarse droplet form, of cholesterol-positive material, and the presence of large numbers of extracellular acicular birefringent crystals. Studies of the adrenal glands of calves which were given DDD for 7.5 to 12.0 weeks showed that bands of collagenous connective tissue occupied the mid-fascicular zones. This was thought to represent an area of interstitial tissue replacement, following parenchymatous degeneration. The remainder of the cortical parenchyma showed essentially no evidence of cytopathogenic effects. These results seemed to indicate that the cytopathogenic effects of DDD on adrenal cortical cells were transitory.

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Epithelial Tumors of the Bovine Gallbladder—A Report of Eighteen Cases

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RESEARCH on tumors occurring in food-producing animals has been limited by the difficulty of collecting adequate numbers for systematic study. The observed occurrence of neoplasia in cattle, sheep, and hogs is low, possibly because relatively few of these animals are permitted to live out their normal life spans and thus attain the comparative biological age at which cancer is most frequently found in man. Abattoirs, where large numbers of slaughtered animals are available for examination, provide an excellent opportunity for collecting material, and much of the tumor research on food-producing animals which has been done in this country has its origin in this source.

In 1950, veterinarians engaged in meat-inspection pathology in the former U.S. Bureau of Animal Industry undertook a comprehensive study of tumors in food-producing animals, based on cases submitted for diagnosis by veterinarians of the Federal Meat Inspection Service. The results of this effort to date have been a paper evaluating the research and economic aspects of tumors,[12] a two-year survey of tumors encountered at the Denver station,[6] and three papers on tumors affecting specific bovine organs or tissues, *i.e.*, adenocarcinoma of the uterus,[8] squamous cell carcinoma of the eye,[7] and multiple schwannomas.[9] This report on epithelial tumors of the bovine gallbladder represents a continuation of the study of neoplasms in food-producing animals, according to species, and with special reference to the site of origin.

The paucity of reports of primary tumors of the gallbladder in cattle suggests that such growths are rare and are to be regarded as pathological curiosities. Feldman[3] describes an adenoma of the bovine gallbladder which arose in the neck of the organ and projected in a papilliferous fashion into the lumen. He also mentions having encountered carcinoma of the gallbladder in his group of 89 carcinomas obtained from cattle. In an earlier case report, Feldman[2] also describes an adenocarcinoma of the gallbladder in a cow, which he considers to be an unusual finding. Davis *et al.*[1] encountered two gallbladder tumors in their series of 90 bovine neoplasms. These were considered to be malignant, and probably are among those included in Feldman's monograph. Trotter,[10] in his series of 300 bovine tumors, list a columnar cell carcinoma of the gallbladder with metastases to the hepatic lymph nodes. Jackson[4] did not have any neoplasms of the gallbladder in his collection of 108 bovine tumors. Adenocarcinomas of

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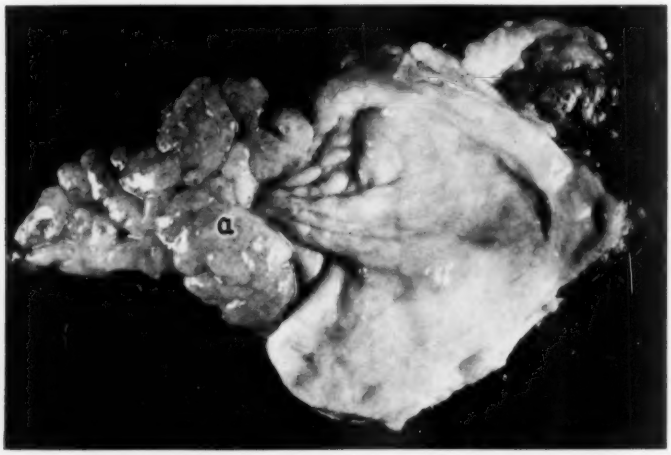


Fig. 1—Papillary cystadenoma (a) arising in the fundus of the gallbladder of a 3-year-old steer. Notice the stalklike attachment to the mucosa.

the gallbladder with lymph node or liver metastases have been reported as individual or small groups of tumors in cattle by European investigators.[5, 10, 11]

In contrast to the limited number of cases recorded in other surveys, Monlux *et al.*[6] encountered ten primary gallbladder tumors in a series of 186 bovine neoplasms (exclusive of ocular tumors) collected in their two-year survey. Nine of these were considered benign, and one malignant; all are included in this paper.

OBSERVATIONS

The 18 epithelial gallbladder tumors described in this series represent meat-inspection specimens which were submitted for diagnosis to the Animal Disease and Parasite Research Division Laboratory at Denver. Sixteen of the specimens were found at the Denver station; the other two originated in federally inspected establishments in California and Oregon, respectively. It is of interest that, of the 16 Denver specimens, 11 were detected by one veterinary inspector. On one occasion, two tumors were observed in one lot of 40 steers and, on another, two tumors were found on the same day, but at different Denver establishments. Seventeen cases were diagnosed as papillary cystadenomas, and one was considered to be a papillary adenocarcinoma.

The 9 animals on which information as to breed was recorded were Herefords. These were all from Denver, however, and since approximately 80 per cent of the cattle slaughtered at this establishment are predominantly Hereford, this should not be taken as evidence of a breed susceptibility. Of the 18 tumors, five were from cows, 11 from steers, and two from aged bulls. This probably does not indicate a predilection for the male, but merely reflects the preponderance of steers slaughtered at this location. The ages of the affected animals were: 2 years, 7; 3 years, 3; 4 years, 2; and 5, 6, and 7 years, 1 each; and 1 cow and 2 bulls were recorded as aged. The condition of the affected animals was reported as good or excellent for 13 animals and as fair for 2 of the aged animals; no record was available for the other 3. Gallstones were found associated with a cystadenoma in only one of the 18 tumors. These were found in the fundus of the gallbladder of a 4-year-old steer and appeared as a collection of small, black, gritty calculi. In 2 other animals, associated liver fluke infestation of the liver was found.

With the exception of the malignant growth, the neoplastic lesions were confined to the gallbladder, and all were found arising from the mucosa of the lumen. Ten of the tumors had their point of attachment in the fundus of the gallbladder, three took origin in the body, and one arose in the neck.

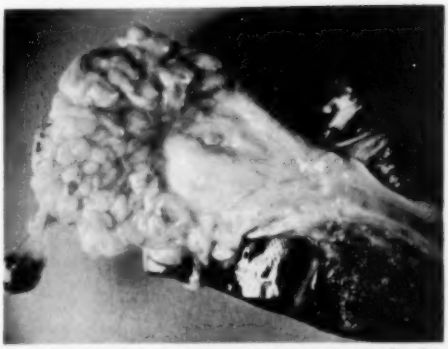


Fig. 2—Papillary cystadenoma arising in the fundus of the gallbladder of an aged bull. Notice the broad area of attachment and the pedunculated cyst.

Characteristically, the papillary cystadenomas appeared as solitary, red or gray epithelial-covered masses, attached to the wall of the gallbladder by a thick, short stalk (fig. 1). The diameter of the basal area of attachment varied from 1 to 4 cm. and, in some instances (fig. 2), assumed a sessile character. Frequently, the surface of the growth formed papillary projections which extended for 2 to 4 cm. (fig. 3). These appeared teatlike or cone-shaped, or sometimes the surface of the growth had a cauliflower-like appearance. Occasional tumors had a rugose character (fig. 4) or a surface raised in smooth, rounded masses

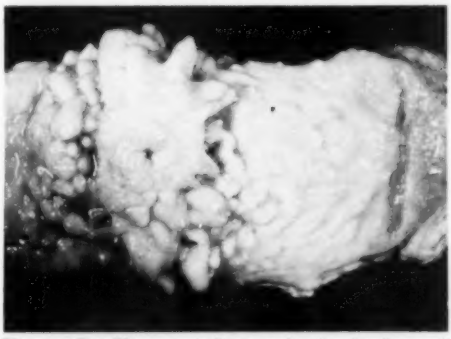


Fig. 3—Papillary cystadenoma in the fundus and body of the gallbladder of an aged cow. The surface of the tumor forms cone-shaped projections.

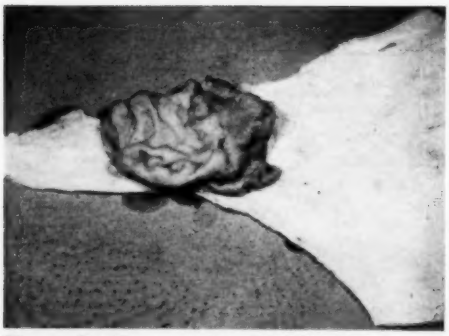


Fig. 4—Papillary cystadenoma arising in the neck of the gallbladder of a 2-year-old steer, formalin-fixed specimen. The surface of the tumor has a rugose character.

and indistinct lobules (fig. 5). Numerous cysts were evident in many of the tumors, and the entire growth was usually of a mucinous character. Frequently, the gallbladders contained considerable quantities of thick, tenacious, mucinous exudate.

The size was variable, but several were approximately 5 to 7 cm. in diameter. Such tumors sometimes distended the gallbladder moderately, occupying most of the lumen and, on occasion, protruding into the neck. The growths were usually detected at postmortem inspection by palpation, or following incision of the gallbladder, since only rarely was there visible external evidence of neoplasia (fig. 6). In this series, the tumors occurred as solitary growths, with one exception. In this case, there were two small, independent tumors arising in the fundus of the organ.

The single case of papillary adenocarcinoma (fig. 7) was manifested by a firm, yellowish, rugose tumor, 4 inches in diameter, having its point of origin midway between the neck and the fundus. The growth had broken through one side of the gallbladder, extensively invaded the adjacent liver tissue, and metastasized to the portal lymph node. In contrast to the 17 benign growths, this tumor was readily apparent on visual examination of the liver and gallbladder.

The neoplastic cells of papillary cystadenoma are recognizable as having their origin in the normal epithelium of the gallbladder. The normal bovine gallbladder is composed of a mucous layer consisting of a surface epithelium and a lamina propria, a layer of smooth muscle, and a perimuscular connective tissue layer, with a serous layer covering part of the organ. The lining epithelium is made up of tall columnar cells with basally placed oval nuclei and a pale, slightly granular cytoplasm. The mucosa is thrown into frequent folds, some of which appear to subdivide and extend deep into the lamina propria. When these are cut at a tangent, they give a false appearance of tubular glands. Within the connective tissue stroma are found groups

Fig. 5—Papillary cystadenoma of the gallbladder of a 2-year-old steer. The surface of the growth has a lobulated appearance.

Fig. 6—Intact gallbladder from an aged bull, which contained a papillary cystadenoma. Despite the knobby appearance of the serosal surface, there was no invasion of the muscularis mucosae.

of tubuloalveolar glands, which appear as small nests. These are most numerous in the region of the neck but also extend, in some degree, throughout the length of the gallbladder to the fundus (fig. 8). They are lined by low columnar cells, many of which have an acidophilic-staining, granular cytoplasm.

The histological pictures in the 17 cases of benign papillary cystadenoma studied in this series are remarkably similar. The surface of the tumor is covered by a single layer of tall columnar epithelial cells, which are continuous with the epithelium lining the adjacent normal gallbladder mucosa. This epithelial covering is thrown into long, branched infoldings, which extend for a considerable distance into the underlying connective tissue stroma and are, in turn, continuous with the papillary, glandlike structures which make up the bulk of the neoplasm. When these tubular alveolar structures are cut in cross section, they appear as regular acinic spaces lying in a loose, edematous connective tissue stroma. Frequently, they are dilated and, sometimes, are greatly distended into cystlike spaces which, in many tumors, are grossly visible (fig. 9, 10). The tall columnar neoplastic cells greatly resemble the

Fig. 7—Papillary adenocarcinoma of the gallbladder of a 6-year-old cow. The primary growth (left), arising in the body of the organ, had broken through the wall and extensively invaded the liver (right).

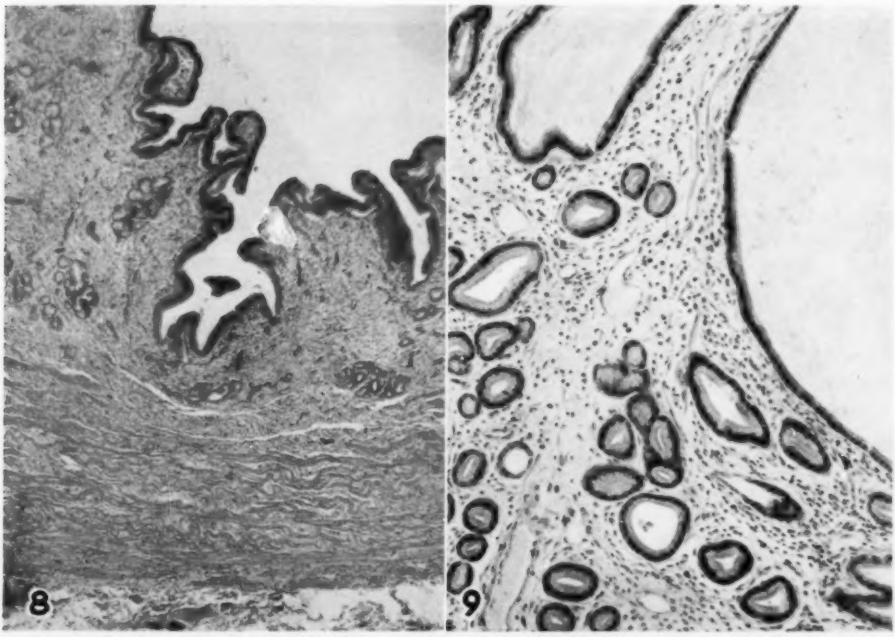
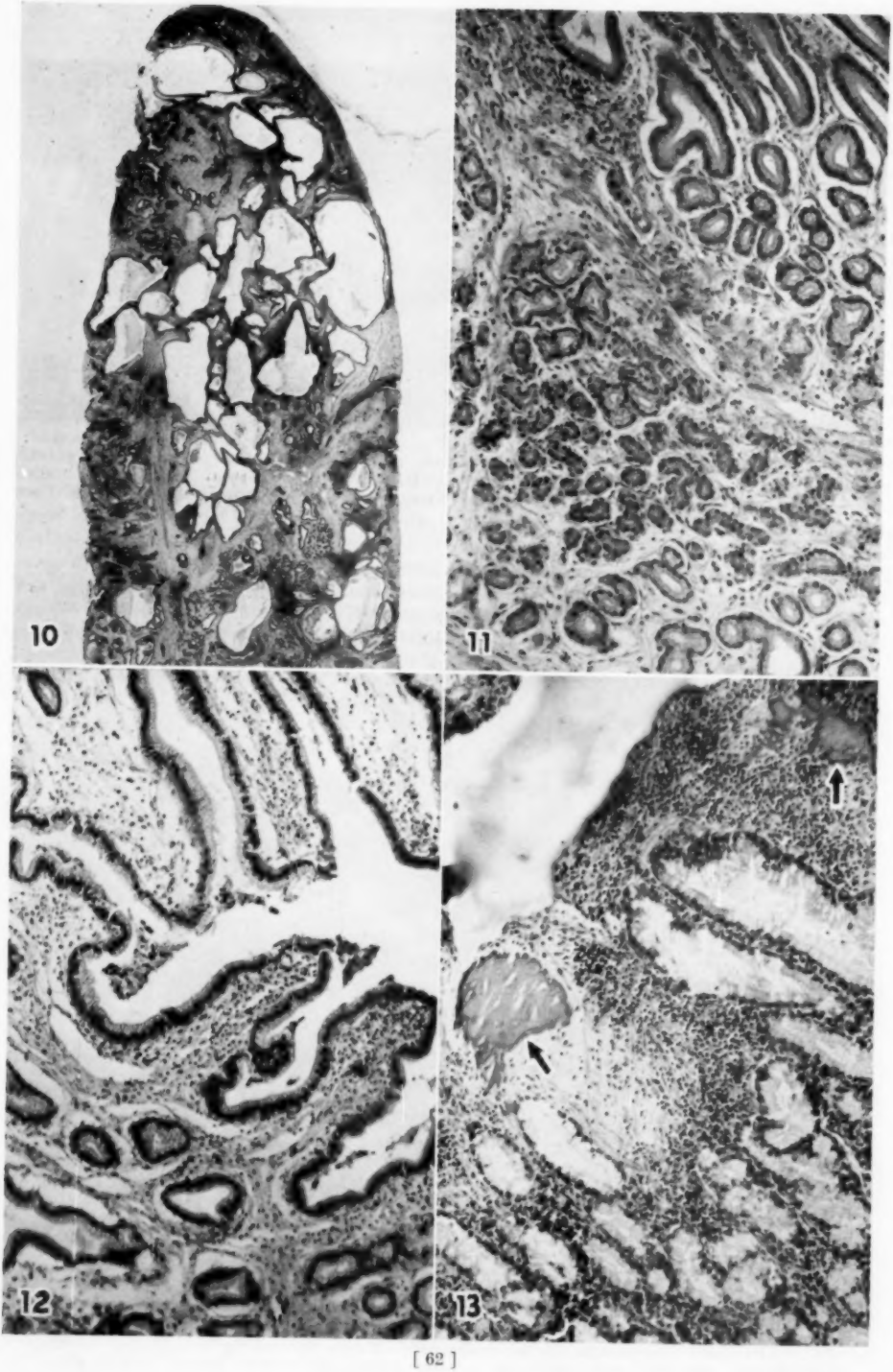


Fig. 8—Section through the wall of a normal bovine gallbladder taken at the fundus. Notice the single layer of tall columnar cells making up the epithelial lining and the scattered nests of glands within the connective tissue stroma. H & E; x 40.

Fig. 9—Papillary cystadenoma of the gallbladder. Notice the orderly arrangement of the tall columnar neoplastic cells which are forming glands and cystlike spaces. H & E; x 110.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epithelial lining of the normal gallbladder mucosa, having a clear cytoplasm and basally placed oval nuclei. They are uniformly arranged in a single layer lining the alveolar spaces, with their deeply-staining nuclei appearing as a dark border around the perimeter.

Deep within the growth, usually close to the muscularis, there is a proliferation of low columnar cells which are also arranged in an adenomatous pattern (fig. 11). The cytoplasm of these cells is frequently granular and stains readily with eosin. They resemble the mucous glands of the normal gallbladder.

The formation of cystlike spaces and the papillary manner of growth (fig. 12) were such a consistent feature in this group of benign tumors that the designation "papillary cystadenoma," was considered justified. The orderly arrangement of the epithelial cells was impressive. It was unusual to find even small groups of anaplastic cells, and mitotic figures either were not found or were demonstrated only with difficulty. There was no attempt to invade and break through the muscular coat although, in one tumor (fig. 6), the expanding growth had caused pressure atrophy and the muscularis was reduced to a thin atrophic layer. In most cases, there was a moderate infiltration by inflammatory cells, chiefly small round cells and eosinophils. Ulceration of the surface was not observed. In the single animal in which gallstones were associated with a tumor, small concretions were found embedded within the wall of the gallbladder, where they had caused some cellular reaction and fibrosis (fig. 13).

The single case of adenocarcinoma in the group bore considerable resemblance to its benign counterpart in the superficial portion of the primary growth, which protruded into the lumen of the gallbladder. Here, the arrangement of the cells forming the acini was comparatively orderly. A

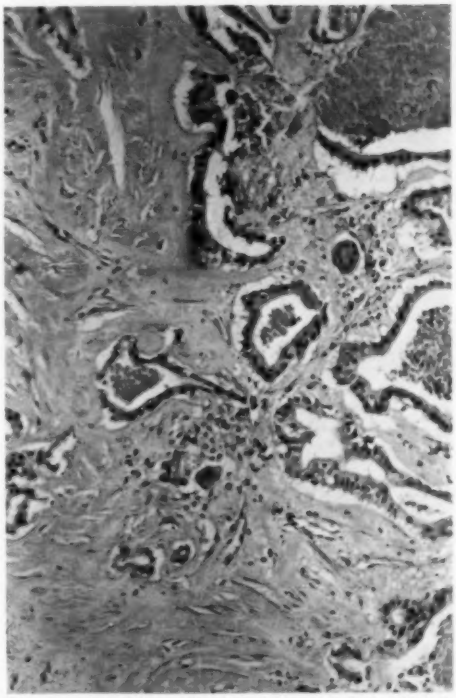

Fig. 14—Papillary adenocarcinoma of the gallbladder. Nests of anaplastic low columnar cells forming glands in a dense connective tissue stroma. H & E; x 110.

papillary pattern prevailed, and the connective tissue stroma appeared loose and edematous. Deep within the tumor and extending into the liver, the growth became more fibrous and nests of tumor cells resembling the low columnar type of mucous glands were found scattered through a dense, collagenous connective tissue matrix. The glandular pattern persisted, but the neoplastic cells were arranged as irregularly shaped groups of anaplastic cells. They frequently formed a stratified epithelium instead of an orderly single layer

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Fig. 10—Papillary cystadenoma of the gallbladder. Section of a single papillary projection taken from tumor in fig. 1, showing cystic character. H & E; x 9.

Fig. 11—Papillary cystadenoma of the gallbladder. A field in which neoplastic cells of both the tall and the low columnar types are evident. H & E; x 110.

Fig. 12—Papillary cystadenoma of the gallbladder. Adenoma showing characteristic papillary arrangement. H & E; x 110.

Fig. 13—Papillary cystadenoma of the gallbladder. Tumor containing intramural concretions (arrows), which were associated with gallstones. H & E; x 110.

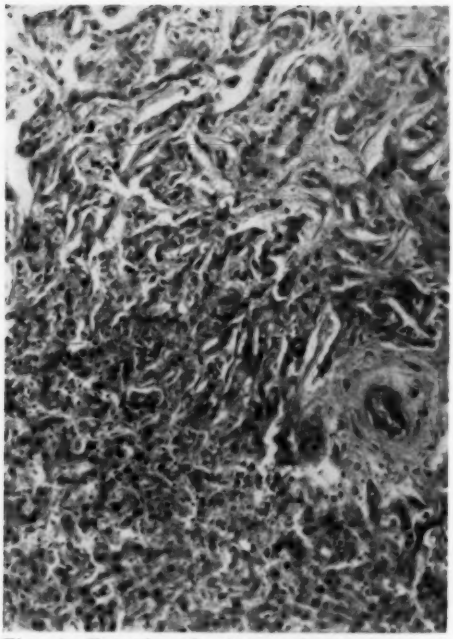

Fig. 15—Extension of primary adenocarcinoma of the gallbladder to the liver. The neoplastic cells are actively invading the liver parenchyma. H & E; x 110.

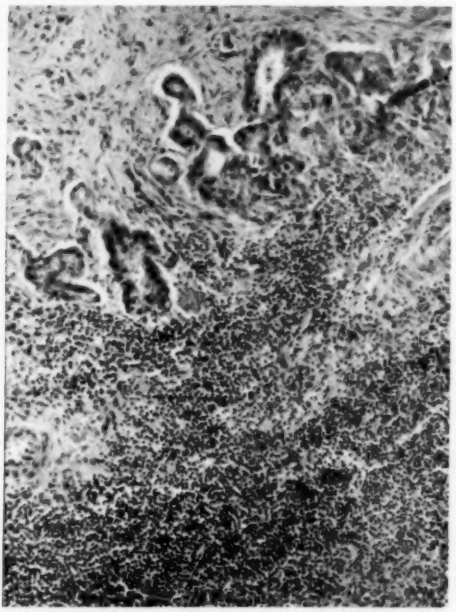

Fig. 16—Metastasis of a primary adenocarcinoma of the gallbladder to a portal lymph node. H & E; x 110.

(fig. 14). Many of the spaces were filled with debris, and scattered focal areas of necrosis, with beginning calcification, were evident. Mitotic figures were readily demonstrated. There was no effort at encapsulation, and the tumor cells were seen actively invading the liver parenchyma (fig. 15). Much of the normal tissue of the hepatic lymph node had been replaced by metastatic tumor growth (fig. 16).

DISCUSSION

Papillary cystadenoma of the gallbladder is not a common tumor in cattle; however, it is probably by no means as rare as the few reports in the literature might suggest. Under routine meat-inspection procedures, the liver and the common bile duct are subjected to careful scrutiny, but there is ordinarily no occasion for the gallbladder to receive more than a cursory examination of the exterior, since this organ is immediately discarded. The gallbladder is somewhat distended with bile and, since these tumors are usually contained within the lumen, they can be detected only by careful palpation or by incision. The fact that 11 of the 18 tumors in this series were submitted by one veterinary inspector is evidence that they may be encountered with relative frequency if a definite search is made.

It is believed that carcinoma of the bovine gallbladder, with extension to the liver, is truly a rarity. Any malignant growth which displayed sufficient invasiveness to break through the wall of the gallbladder and penetrate the liver could hardly escape detection at postmortem inspection. However, the single case of papillary adenocarcinoma of the gallbladder was the only one received during 24 years of meat-inspection diagnosis at the Denver laboratory.

In none of the benign cases did the affected animals show any associated lesions or signs which could be attributed to the tumor. It is probable that benign tumors of the gallbladder do little harm unless they attain such size that they cause mechanical interference with the biliary system. Invasive malignant growths, however, are prone to penetrate the liver in the region of the portal fissure, thereby endangering the large vessels and ducts.

Differentiation between a benign process and one showing low-grade histological malignancy is difficult, and distinctions must, of necessity, be somewhat arbitrary. The orderly arrangement of the neoplastic

cells and failure to invade the muscularis mucosae were used as bases for considering 17 of these tumors to be benign adenomas. Whether any of the cystadenomas in this series might have become malignant, had the animals been permitted to live out their normal life spans, can not be resolved by our observations. The fact that 12 of the 18 animals were under 5 years of age suggests that these growths frequently develop early in life, and the apparent rarity of frank adenocarcinoma indicates that malignant transformation occurs infrequently.

Gallstones were found associated with only one case, and it is probable that cholelithiasis is not an etiological factor of importance. Liver fluke infestation in 2 animals was also considered an incidental finding.

Neoplasms of the bovine gallbladder do not represent a problem of serious economic or clinical importance. However, papillary cystadenoma of this organ is believed to represent a distinct pathological entity, which occurs with some regularity and must be given recognition in the consideration of the subject of neoplasia in domestic animals. Monlux *et al.*[6] have pointed out that, while most of the important types of tumors will be encountered in the routine diagnosis of meat-inspection specimens, it is hazardous to attempt to estimate their relative occurrence on the basis of the comparative number of cases submitted. Papillary cystadenoma of the gallbladder is an example of a tumor which, because of its unobtrusiveness, has largely escaped the attention of veterinary pathologists.

SUMMARY

Eighteen primary epithelial tumors of the bovine gallbladder are described. Of these, 17 were considered to be papillary cystadenomas, and one a papillary adenocarcinoma, with extension to the liver and portal lymph node metastasis.

Evidence is presented which suggests that papillary cystadenoma of the bovine gallbladder occurs with more frequency than is suggested by the few reports in the literature. Adenocarcinoma of this organ is believed to be rare.

Neither cholelithiasis nor liver fluke infestation appears to have a role in the etiology of papillary cystadenoma.

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The Cytopathogenesis of Vesicular Stomatitis Virus Infection in Cattle

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VESICULAR STOMATITIS is a virus disease which naturally affects cattle, swine, horses and, probably, deer and raccoons.[14] In cloven-hoofed animals, vesicles or ulcerations occur in the epithelium of the tongue, gums, hard palate, lips, teats, udder, coronary and interdigital regions and, in swine, also on the snout. The disease occurs sporadically in the northern United States, but is enzootic in the southeast,[21] in Mexico, and in central and northern South America.[12]

Although the pathological changes occurring in cattle affected with vesicular stomatitis have been described,[4] it was believed that a more extensive study of the nature of their development might give an indication as to how the virus enters susceptible tissue. Similarly, a study of the cellular pathogenesis of this disease might explain the reason for the vesicular response to this virus, rather than the more common cellular changes associated with other virus infections. Lastly, in order to understand more fully the nature and overall pathogenesis of the vesicular diseases, it seemed desirable to attempt to correlate morphological changes in affected tissues with entry, multiplication, and egress of the virus. It was with these objectives in mind that this study was undertaken.

REVIEW OF THE LITERATURE

The first published description of vesicular stomatitis appears to be that of Theiler,[26] who described cases in South African horses occurring in 1884 and 1897. It was described by Teidebold *et al.*[27] in 1916, as affecting horses in the United States. Although in the following year Eichhorn[9] described its occurrence in cattle, the disease is still referred to in many veterinary texts primarily as a disease of horses. It has since been demonstrated in naturally affected swine[21] and in people working with the experimental disease.[12] Neutralizing antibodies have been demonstrated in deer and raccoons, and the disease has been produced experimentally in these animals.[14]

Cotton[5] demonstrated the viral nature of the disease, and the causative organism has since been shown to multiply in a number of experimental animals.[6, 7, 15, 16, 24, 28, 29] The virus has likewise been shown to multiply in the embryonating hen's egg[2] and in tissue culture.[1, 3, 6, 23] Studies have indicated a number of possible means of transmission, but none have determined beyond doubt its means of spreading in nature. The similarity of this disease to foot-and-mouth disease led early workers to suspect a similar means of transmission, *i.e.*, by direct contact or by means of contaminated environment. However, Theiler[26] was unable to infect susceptible horses watered from the same bucket as infected horses unless the former previously had frank abrasions on the mouth parts. Olitsky *et al.*[17] exposed 6 cattle by contact with 6 infected animals, and only 3 of the former became infected. Wagener[30] was unable to infect calves by placing them with infected cattle. The lack of spread between contiguous farms, the occurrence of the disease only in wet or wooded regions, and its disappearance with the onset of fall frosts caused Hanson[12] to propose that the disease was spread by insects. Ferris *et al.*[10] showed that certain Diptera were capable of transmitting the disease mechanically.

The initial lesion in vesicular stomatitis of cattle is a raised, flattened, pale pink to blanched papule in or near the mouth parts which, in natural infections, may develop in from 18 hours to five days.[25] In experimentally inoculated animals, the initial papule may be detected about 18 hours following inoculation.[4] Mammary erosions or, more frequently, foot affections, such as lameness, are the first abnormalities seen. However, when the mouths of such animals are examined, oral vesicles or ulcerations are invariably present. The initial localized pale elevation is a few millimeters in diameter, but rapidly becomes a pink, inflammatory papule. It may occur on the tongue, gums, hard palate, lips, buccal mucosa, or muzzle. The area becomes larger and reddened, and drooling, swelling of the nostrils, and sensitivity to handling occur. Within a day or two, the flattened papule becomes a vesicle up to 2 or 3 cm. in diameter. Around the vesicle new vesicles appear, which coalesce and may involve a large area. They usually rupture before the second day and discharge clear, watery fluid; a shallow ulceration results. The epithelial tissue regenerates quickly and a thin, pale gray zone of ingrowing epithelium may be detected within 48 hours following ulceration. Within one or two weeks, healing is com-

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plete, and the site of ulceration is not apparent. If secondary infection occurs, the contents of the vesicle are purulent and a scar may remain. However, secondary infections are exceptional.[4]

Chow *et al.*[4] have described the histological changes of the epithelium as beginning in the stratum spinosum. The intercellular bridges became stretched, and spongy cavitation appeared between the cells. As the process continued, these intercellular vacuoles expanded as the cells decreased in size and pulled apart. These spaces were apparently filled with fluid, as they eventually coalesced to form the vesicle in which the detached affected cells floated as their cytoplasm disintegrated. Nuclear changes were not described, but became evident after cytoplasmic disintegration and, eventually, the nucleus disappeared. In the final stages, the process extended to the basal layer, but the latter was generally left intact. Extravasation of erythrocytes and infiltration by neutrophils generally occurred, to a limited extent, in the vesicles. The upper portion of the dermis (in the mouth, the lamina propria) became edematous and adjacent muscle sometimes became inflamed. The vesicle then ruptured and, shortly thereafter, new epithelium grew both upward and centrally from the persisting basal layer. Inclusion bodies were not seen in affected epithelium.

Studies on the rate and manner of multiplication of vesicular stomatitis virus have not been extensive. Sorensen[25] was able to isolate virus from the saliva of intralingually inoculated cattle within 24 hours following inoculation. At about the same time, virus could likewise be demonstrated in the blood. Cotton[6] demonstrated virus in the blood of experimentally infected horses and cattle, 24 hours following inoculation of the tongue, and persisting up to 66 hours following inoculation. Shahan *et al.*[23] demonstrated virus in the blood of experimentally inoculated swine three and four days after the appearance of the primary vesicles. Virus has never been isolated from urine and feces[15,25] and is rarely isolated from saliva once healing has begun.[25] Transmission by direct contact has seldom been successful, and then only when vesicles are present, and up to about five days following infection (about 3 days following vesiculation). The saliva is infectious during this period.[6,15] Tissue from infected swine has proved to be infectious up to six days following vesiculation.[18]

Growth curve studies of Timm,[29] using surviving cultures of chicken chorioallantoic membranes, showed an initial rise between the second and fourth hours, of roughly one half log of virus, from an inoculum of 10^4 or 10^5 egg infective doses of vesicular stomatitis. The titer reached its peak at about the eighth hour of incubation. Bachrach *et al.*,[2] in tissue culture studies using swine kidney epithelium and calf tongue epithelium, reported the highest titer, as well as the maximum number of cells destroyed, as occurring at 48 hours postinoculation. Changes were described as rounding of epithelial cells, pulling away from adjacent cells, shrinkage and, eventually, fragmentation. Grossly visible foci of necrosis may be seen when the virus is inoculated onto monolayer cultures of calf and swine kidney.[28]

MATERIALS AND METHODS

The virus used was the Wisconsin strain, New Jersey type of vesicular stomatitis virus.* The virus was stored as infected allantoic fluid at —20 C., with occasional egg passages. At the beginning of this study, it was verified by serum-neutralization tests to be New Jersey type vesicular stomatitis virus and then passed in guinea pigs to verify its mammalian pathogenicity. Footpad vesicle fluid was then passed once by inoculation into the allantoic sacs of 8-day embryonating chicken eggs. The fluid from this passage was pooled, stored in screw-cap Pyrex tubes at —20 C., and aliquots were used as the virus for this study.

Intact pregnant bovine uteri were obtained from a local slaughterhouse and, within one hour of slaughter, the fetal skin was removed. Tissue cultures were prepared by scraping, with a razor blade, the outermost epithelial layers from the skin of bovine fetuses less than 5 months old. The skin was placed in chilled Hank's solution[11] and minced with iris scissors until pieces roughly 1 mm. square were obtained. The fragments were allowed to settle, then were rinsed once with Hank's solution. This solution was then replaced by 0.5 per cent trypsin in Hank's solution, the pH was adjusted to 7.2 with 0.1 *N* sodium hydroxide, and the suspension incubated at 37 C. for 20 minutes. This short period was sufficient to allow the adherent dermal connective tissue to digest and be removed. The supernatant fluid was discarded and the epithelial residue washed twice with Hank's solution, then passed through a 20-mesh stainless steel screen. This was resus-

* Originally isolated by Dr. C. A. Brandly from ulcerative lesions of a bovine mouth during the 1949 Wisconsin epizootic.

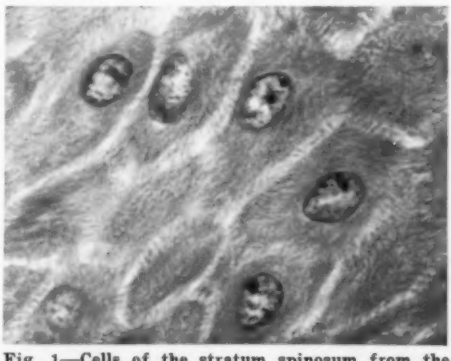

Fig. 1—Cells of the stratum spinosum from the tongue, showing the earliest visible changes following infection of the calf with vesicular stomatitis virus. Notice the widened intercellular spaces and prominent intercellular bridges. H & E; x 326.

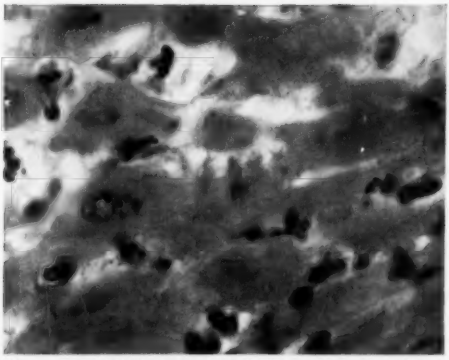

Fig. 2—Infected bovine epithelium, with prominent contraction of cytoplasm and increasing amounts of intercellular fluid. H & E; x 351.

pended in trypsin, at pH 7.2, and incubated for one hour at 37 C. This final cell suspension was passed through four layers of sterile cheesecloth and then washed three times with Hank's solution. The cells were then resuspended in the medium of Reissig and Melnick,[20] modified to the following composition:

Lactalbumin enzymatic hydrolysate.....	0.5%
Gamma-globulin-free calf serum.....	2.0%
Hank's salt solution.....	97.5%
Penicillin, crystalline G.....	100 units/ml.
Dihydrostreptomycin sulfate.....	50 μg./ml.
Phenol red (1% aqueous).....	1 drop/10 ml.

The pH of the medium was adjusted to 7.2 with 0.1 N sodium hydroxide.

Culture vessels consisted of 16-cm. lengths of 3.5-cm. Pyrex tubing, sealed and flattened at one end and along the bottom. About 2 cm. from the open end, a raised inner lip was formed in the lower flattened surface, to prevent outflow of medium during incubation. Three 21-mm., No. 1 microscope coverslips (slips) were aligned along the bottom of the vessel and fixed with heparinized chicken plasma, clotted with chicken embryo extract in Hank's solution. As soon as the plasma clot was well formed, 7.2 ml. of the previously described cell suspension was pipetted into the vessels and the vessels were stoppered with rubber corks. Gassing was not necessary. The slip-containing flasks were incubated at 37 C. in a horizontal position. The cells settled across the entire lower surface of the flask, some upon the slips. Only rough efforts were made to standardize the cell suspensions, since the number of cells settling onto the surface of the slips varied from experiment to experiment.

By the fourth day of incubation, a thin sheet of skin cells had grown across the slips. A single slip was then removed, rinsed in warm Hank's solution, and placed with the cells uppermost in a small Petri dish. Warmed, virus-infected allantoic fluid (0.8 ml.) was placed on the slip, and it was covered and allowed to stand for 15 minutes. Control culture slips were treated with virus neutralized with equal parts undiluted homologous serum at 37 C. for 30 minutes, and also with virus heated at 55 C. for 30 minutes.

The treated slip was placed, with the cells down, over a stainless steel plate, the size and shape of an ordinary microscope slide, but with an 18-mm. hole drilled through the center. The edges of the slip were sealed with a warm mixture of 3 parts paraffin to 1 part petrolatum. The plate was quickly inverted, the hollow center filled with warmed medium, and a clean slip was fixed to the opposite side.

This virus-infected culture plate was placed on a warm stage (37 C.) of a microscope equipped with dark medium phase objectives. The course of cellular changes was observed and photographed using 35-mm. Du Pont micropy film and a No. 11 green filter.

Growth curve studies were performed using the previously described cultures in the tubular Pyrex flasks. The old medium in the flasks was replaced by a solution containing 0.8 ml. of infected allantoic fluid in 7.2 ml. of medium. Hourly, 0.3-ml. samples were removed and replaced by 0.3 ml. of new medium. Samples from two series of parallel cultures were pooled, diluted tenfold through 10^{-7}, and 0.1 ml. of each dilution was inoculated into the allantoic sacs of 6, 8-day embryonating chicken eggs. The eggs were candled daily and all deaths recorded. The titer of each hourly sample was expressed as the $e.l.d._{50}$, as calculated by the procedure of Reed and Muench.[19] Four such growth curve studies were made.

Histological studies were performed on 2 calves, 2 weeks old, inoculated intralingually with the same virus as the cultures. One calf was killed 24 hours following inoculation, when the lingual vesicles were apparently at their maximum. The second was allowed to recover so that any generalization might be observed; however, there was none. In addition, material was available from previous studies in this department on the same strain of bovine vesicular stomatitis.[4] In the latter study, tissue was available from calves killed 20, 40, and 100 hours after inoculation. All tissues were fixed in 10 per cent formalin, embedded in paraffin, and stained with Harris' hematoxylin and eosin Y.

RESULTS AND DISCUSSION

Histopathology of Vesicular Stomatitis in the Calf Tongue.—In lingual tissues, removed at the specified periods, a sequence of characteristic changes in the epithelium could be recognized. The first changes were detected in the deeper layers of the stratum spinosum (prickle cell layer). Apparently simultaneously, there was an increasing prominence of the intercellular spaces and a stretching of the intercellular bridges (fig. 1), accomplished by a concomitant re-

duction in the volume of cytoplasm of the affected cells. Initially, the internal morphology of the cells was not altered as the cytoplasm decreased; the homogeneous, finely granular character of the cytoplasm persisted.

Occasionally, in the midst of a group of epithelial cells showing early cytoplasmic shrinkage, a cell would be found which had a ballooned, brightly eosinophilic cytoplasm. The significance of this occasional change was never determined.

The intercellular spaces became wider and the connecting processes more extended until the latter finally gave way. A state of epithelial spongiosis then existed (fig. 2), followed rapidly by a contraction of the cytoplasm about the nuclei until only a narrow rim of cytoplasm remained, giving the cells the appearance of large lymphoblasts. By this time, the affected cells had lost all attachment and were floating freely in the enlarging vesicle, surrounded only by a thin, spongy reticulum of cytoplasmic remnants (fig. 3). The amount of intercellular fluid was greater than could be accounted for by the fluid component of degenerating cytoplasm and

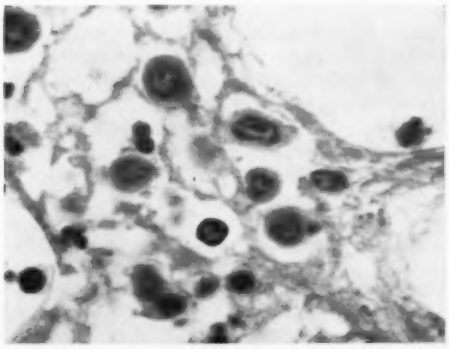

Fig. 3—Infected bovine epithelial cells with severe cytoplasmic contraction and unaltered nuclei. Affected cells now float free in the fluid of the vesicle and resemble large lymphoblasts. H & E; x 325.

must have been, primarily, an inflammatory exudate. It contained only limited amounts of the fibrin normally encountered in serous exudates, so must represent an extremely low specific gravity exudate, perhaps almost a transudate. At no time was the swelling and cytoplasmic droplet formation, that characterizes hydropic change in epithelial cells, seen in affected cells.

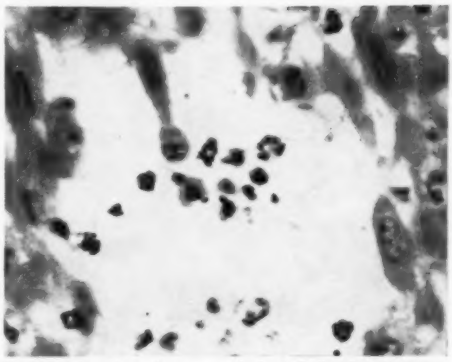

Fig. 4—Section from an infected bovine tongue. The fully developed micro-vesicle contains edema fluid, cellular debris, and neutrophils. H & E; x 351.

Up to this stage, the nucleus had maintained its integrity, and no detectable changes had been seen in the face of this obvious cytoplasmic disintegration. The nucleus then began to contract and, with its thin rim of cytoplasm, eventually formed a small, granular, free-floating mass, which was difficult to distinguish from the neutrophils which simultaneously entered the area (fig. 4).

The fluid content of the vesicle increased, as did the number of neutrophils, until a large, fully-developed vesicle existed (fig. 5). The process generally involved the basal layer of epithelium and extended up to the lower surface of the stratum corneum. Edema of the underlying dermis was common and inflammatory changes, such as hyperemia, edema, and moderate neutrophil infiltration, were visible in the musculature immediately beneath the dermis. The contents of the vesicle were never grossly purulent prior to rupture of the vesicle.

In studies of epithelial changes in the guinea pig, many obvious, small, spherical, eosinophilic nuclear inclusions were seen. However, on only one or two occasions were such structures suspected in affected bovine epithelium; their presence was considered dubious.

Growth Curve Studies.—A series of four growth curve experiments were performed. In the final experiment (fig. 6), the first liberation of virus from infected bovine epithelium occurred at about the third hour after infection. Tissue culture studies were directed particularly at this early

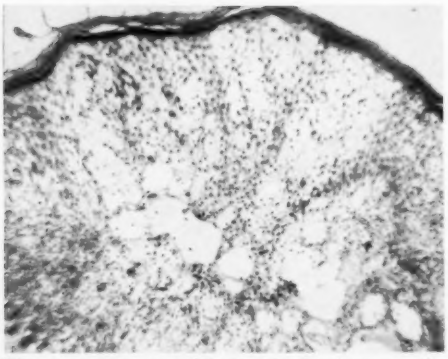

Fig. 5—A mature vesicle in a bovine tongue, prior to final coalescence of micro-vesicles. H & E; x 36.

period. No effort was made to determine the peak or termination of virus growth.

The Pathology of Vesicular Stomatitis in Tissue Culture.—The changes induced by vesicular stomatitis virus in tissue cultures derived from bovine skin were surprisingly similar to those encountered in the infected host animal. Originally, it was believed that certain of the changes in the infected host's cells might be due to secondary effects rather than to direct viral action; *i.e.*, the pressure of the vesicle fluid upon the cell; the effect of toxic products from necrotic tissue; or the effect of secondary inflammatory processes, such as the increased blood flow in hyperemia; action of leukocytes; or the effect of lymph stasis. However, the basic cellular changes occurred both in the absence and presence of these possible complicating factors.

Changes in affected cells could be detected early. At, or soon after, the second hour, the cytoplasm of the cell was seen to be contracting. Short, stubby cytoplasmic processes were present, and appeared to secure the cell to its glass foundation. They were probably homologues of the intercellular bridges in the intact stratum spinosum. These processes continued to adhere to the glass but, as the cytoplasm contracted about them, they became stretched and increasingly prominent. Dozens of small, spherical, highly refractile bodies were usually present in the central portion of the cytoplasm about the nucleus. These were sudanophilic and osmophilic and were considered to be Golgi apparatus. They were unaltered in appearance. In the normal cell, at any given time, roughly a dozen elongated filamentous mitochrondria could be seen. Their morphology and distribution, in a manner radiating outward from about the nucleus into the processes, and the fact that they retained Janus green B in the green (oxidized) state indicated their mitochondrial nature. At two hours, they were unaltered in appearance, and the nucleus appeared normal.

Four hours following infection, the cytoplasm had shrunk noticeably, although the extended processes remained, thus giving the cell a tentacled or jelly-fish appearance. At this time, the mitochondria had developed globular, highly refractile swellings along their length, or were beginning to contract into small spherical masses resembling Golgi apparatus. The nucleus remained unchanged.

By the sixth hour, the cytoplasm had contracted and its processes were retracted until only a few surface irregularities indicated the previous presence of these processes. The cytoplasm now occupied a volume roughly one fourth its original size. The mitochondria had contracted into small, dense, highly refractile bodies, indistinguishable from the Golgi apparatus, and they were no longer recognizable. About this time, the nucleus began to decrease in size, although its membrane and internal chromatin masses appeared unaltered. The natural rigidity of the nucleus or its limiting membrane seemed to be decreasing, since it would now show temporary irregularities in its border as cytoplasmic constituents pushed against it.

The eighth hour changes were an extension of those encountered earlier. The cytoplasm now formed a small spherical mass enclosing the shrinking nucleus. No cytoplasmic constituents were distinguishable; only small irregular granules could be seen with the cytoplasm. The nucleus was now roughly one third its original size, but re-

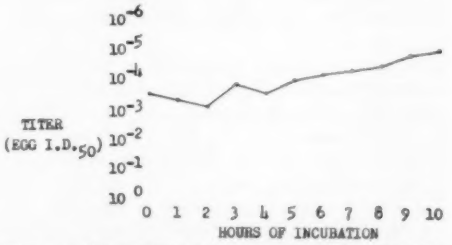

Fig. 6.—The growth curve of vesicular stomatitis virus in cultures of bovine skin epithelium.

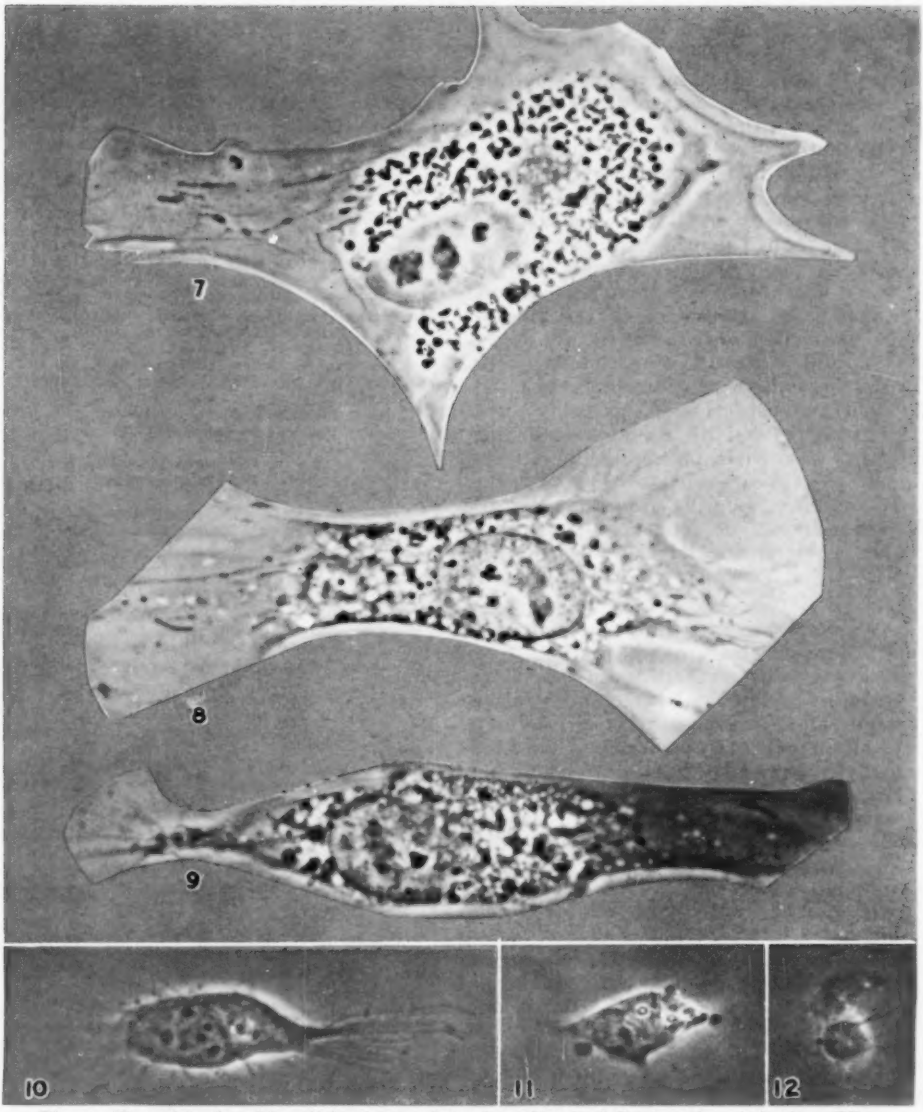

Fig. 7—Normal bovine skin cell in tissue culture immediately following virus infection. Filamentous bodies to the left of the nucleus are mitochondria; dense spherical particles are Golgi apparatus. Dark medium phase; x 1,490.

Fig. 8—Cell of fig. 7 two hours following virus infection. Cytoplasmic contraction has begun, filamentous processes are prominent, mitochondria are unaffected. Dark medium phase; x 1,490.

Fig. 9—Cell of fig. 7 four hours following infection. Cytoplasmic contraction continues, cellular processes are still visible, mitochondria are indistinct. Dark medium phase; x 1,490.

Fig. 10—Cell of fig. 7 six hours following infection. Severe cytoplasmic contraction is seen; the nucleus is unaltered. Dark medium phase; x 1,490.

Fig. 11—Cell of fig. 7 eight hours following infection. The cytoplasm is barely recognizable. Nuclear shrinkage has begun. Dark medium phase; x 1,490.

Fig. 12—Cell of fig. 7 ten hours following infection. All that is seen is the shrunken residual nucleus. At this point, the cell lost all attachment and floated free in the medium. Dark medium phase; x 1,490.

tained its well-defined membranous outline and the characteristic large, rough chromatin granules within.

The tenth to twelfth hour changes indicated the final fate of the cell. Cytoplasmic shrinkage continued; nuclear contraction increased greatly until the nucleus was only a small, ball-shaped mass, bounded by the more prominent spherical mass of granular cytoplasm. At this point, the cell lost its remaining attachment to the culture slide and floated away in the culture tissue. The number of free-floating shrunken cells in the vesicles, seen in infected animals at 20 hours, indicates that it is probably about this period of ten to 12 hours when cells begin to lose their intraepithelial attachment. It appears obvious that, by the twelfth hour following infection, the affected cell can no longer be considered either viable or functional.

SUMMARY

Vesicular stomatitis infection of bovine epithelium is manifested, initially, by changes in the stratum spinosum, later extending to the basal layer and stratum granulosum. The virus effect begins as a contraction of the cytoplasm of infected cells, concomitantly with increased prominence of the intercellular bridges. Cytoplasmic shrinkage continues until the cell consists of an apparently unaltered nucleus surrounded by a thin rim of cytoplasm. Nuclear pyknosis then occurs, and the cell floats freely in the accumulated vesicle fluid.

Studies of cultures of infected bovine skin revealed that release of virus from infected cells began about the third hour following infection.

In phase microscope studies of infected bovine skin in tissue culture, basically the same changes were encountered as in the infected host. About two hours after infection, before virus liberation could be detected, contraction of cytoplasm was seen, along with continued extension of the cytoplasmic processes. By the fourth hour, the filamentous mitochondria had developed globular swellings. Cytoplasmic contraction continued and, by the sixth hour, mitochondria were no longer detectable. The nucleus then began to shrink, although at a slower rate than did the cytoplasm. The process was complete by the tenth to twelfth hours, at which time the cell floated free as a small, spherical mass of granular cytoplasm enclosing a minute, shrunken residual nucleus. The viability of vesicular stomatitis-infected bovine epithelial cells is, therefore, considered to be less than 12 hours.

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Persistence in Cattle of Serum-Neutralizing Antibodies of Vesicular Stomatitis Virus

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FOLLOWING INFECTION with vesicular stomatitis virus, cattle develop a measurable immunity which is demonstrable in three ways: refractoriness of the animal to infection, capacity of the serum to neutralize virus *in vitro*, and capacity of the serum to fix complement in the presence of virus.

Refractoriness is usually of short duration. Sorensen[7] has reported that 2 of 4 cows, exposed a month after their initial infection, developed typical lesions, although high virus-neutralizing titers were demonstrable in the serum of the animals at the time of the second exposure. Shahan[6] summarized his observations on vesicular stomatitis in cattle by stating that measurable resistance is, in some cases, of short duration.

This study concerns the persistence of virus-neutralizing antibodies in more than 50 cows. The antibody titers of 6 inoculated animals were determined at frequent intervals for two years, and the titers of more than 40 infected animals were determined at less frequent intervals over a period of four years.

MATERIALS AND METHODS

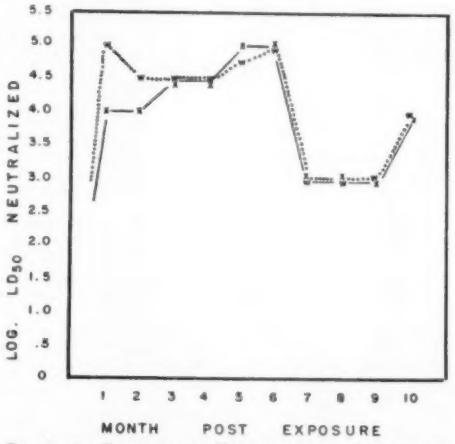
Vesicular stomatitis virus, New Jersey serotype,[2] isolated in Wisconsin in 1949, was used to infect and challenge cattle, and to determine the neutralizing activity of the serums. Pools of allantoic fluids from embryos infected with the tenth to fourteenth egg passages of virus were frozen at −20 C. and served as the source inoculums for all tests. These fluids had an infective titer of 10^5 to 10^4 per milliliter for cattle when inoculated into the tongue epithelium, and a lethal titer of 10^4 to 10^7 per milliliter when inoculated into 8-day-old chicken embryos.

The experimental cattle were obtained in the vicinity of Madison, Wis., and did not possess neutralizing antibodies to vesicular stomatitis virus prior to inoculation. At the time of exposure and for a period thereafter, the animals were confined in an isolation barn and observed daily. A month after exposure, the animals were transferred to a holding barn. Blood was obtained once a month from the jugular vein, the serum separated, and stored at −20 C. Serum was inactivated at 56 C. for 30 minutes prior to use in neutralization tests, which were carried out in 8- or 9-day-old embryonating eggs. Serial tenfold dilutions of the virus were prepared in tryptone broth, and equal amounts of viral dilution and undiluted serum were mixed and incubated for 30 to 60 minutes at room temperature. The allantoic sacs of four to six embryonating eggs were injected with 0.1 ml. of each mixture. The inoculated eggs were incubated and candled twice daily for four days. Survival was accepted as evidence of neutralization.

RESULTS

The first part of the study dealt with the persistence of serum-neutralizing antibodies in experimentally infected cattle.

Experiment 1.—Two cows (x, w), in-



Graph 1—Serum-neutralizing titers of cows X and W.

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fected by the inoculation of virus into the dorsum of the tongue, developed typical lesions in 48 hours. Healing was fairly complete in ten days. The recovered animals were removed to a holding barn, 50 yards away on the same farm, and were kept confined for the duration of the study. Between the exposure and holding barns there was a cattle barn in which 35 milking cows were stabled. The animals in the holding barn were always fed and watered before any other stock on the premises. On several occasions during the period of study, the exposure barn was used for experiments in which animals were infected with vesicular stomatitis virus.

Serum-neutralizing titers of the 2 cows were determined each month (graph 1). A rise of titers at the ninth month was believed to be evidence of failure of the isolation facilities. Accordingly, the experiment was terminated.

Experiment 2.—Four cows (A, B, C, D), 2 of them pregnant, were inoculated on the dorsum of the tongue with the egg-propagated virus. Typical lesions developed within 48 hours. Healing was complete in about two weeks. These animals, like those in the first group, were moved from the exposure barn to the holding barn. At this time, additional precautions were taken to avoid re-exposure of the animals. Virus was not brought to the farm during the remaining period of the study. Neutralizing titers were ascertained at monthly intervals (graph 2).

As in the first experiment, the serum-neutralization titers reached a peak within two weeks, remained high for two months, then fell slowly. A low was reached in about five months. A second peak developed at eight months, a third at 14 months, and a fourth at 23 months. There was no evidence that infection of these cows was brought from outside sources. The parallel between experiments 1 and 2 was believed to be significant.

Natural Infection.—The persistence of neutralizing antibodies was also followed in naturally infected cattle. A herd of about 30 cows, on a farm near Bruce, Wis., developed signs of vesicular stomatitis in September, 1949. They were observed by Dr. J. E. McDermid, the local veterinarian. Blood samples were drawn one, six, and 46 months after infection, and serum-neutralization titers were determined (table 1).

TABLE 1—Neutralizing Antibodies for Vesicular Stomatitis in a Herd of Cows Near Bruce, Wisconsin

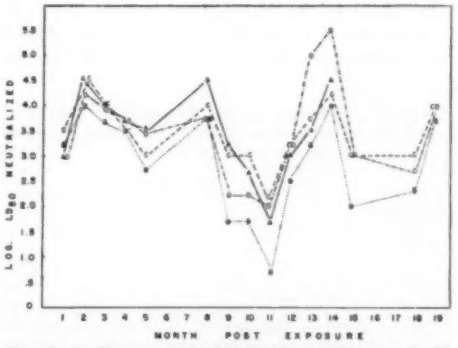
Month	No. serums tested	No. negative	No. positive	Titers of positive serums *
1	1	0	1	3.2
6	25	24	1	2.5
46	8	2	6	3.0, 3.7, 4.0, 4.0, 4.0, 4.0

* Titer expressed as the logarithm of the l.d.$_{50}$ virus neutralized.

Nine other herds in the vicinity of Bruce, Wis., present in the area of the 1949 epizootic, were bled 40 to 46 months after the passing of the epizootic. Serum-neutralization titers, as determined for these samples, are recorded (table 2).

Three years after the epizootic, 48 of 73 blood samples, obtained from cattle that were part of the infected herds, contained neutralizing antibodies. Only one of the 32 blood samples obtained from animals born since that time, in the same herds, contained neutralizing antibodies. The one positive sample came from a 3-month-old calf born in January, 1954. Since the 2 calves of experimentally infected cows (experiment 2) possessed neutralizing antibodies for several months after birth, it appeared possible that this calf of an antibody-carrying dam may have retained colostral antibodies for three months.

Blood samples were taken from the calf at 7 months of age, and the serum at this time did not contain antibodies. Samples taken from the dam at the same time, four



Graph 2—Serum-neutralizing titers of cows A, B, C, and D.

TABLE 2—Serum-Neutralizing Titers of Cattle in the Vicinity of Bruce, Wis., at 40, 46, and 52 Months After the Epizootic of 1949

History of animal	Month blood samples taken	No. cows sampled	Titer groups *					Negative (%)	Positive (%)
			0	1	2	3	4		
Born prior to 1949									
Lesions seen	40	21	8	0	2	1	10	38	62
	46	32	6	1	1	10	14	22	78
No lesions seen	40	9	5	2	0	0	2	78	22
	46	11	3	2	2	0	4	46	54
Born since 1949	40	4	3	1	0	0	0	100	0
	52	28	27	0	0	1	0	97	3

* Titer expressed as the logarithm of the l.d.$_{50}$ virus neutralized.

and one half years after infection, had a serum-neutralization titer of 10^4. While the neutralizing antibody titer of the calf was shown to be transitory, as that of passive antibody, titers of infected animals have also disappeared in a short time (table 3). The persistence of colostral antibody in significant titer, for three months and possibly longer, is difficult to explain on the basis of other studies, particularly those of McDiarmid,[5] who worked with the transference of agglutinins for *Brucella abortus* from cow to calf, and found that agglutinin titer usually disappeared six weeks after birth. However, Binns,[1] working with Rinderpest in Kenya, found that many calves retained, for three to four months, sufficient colostral antibody to interfere with satisfactory vaccination.

The virus-neutralizing capacity of the serums from both experimentally infected and naturally infected animals appeared to fluctuate in cycles lasting several months (graph 2 and table 3) without a significant long-term loss in titer. The absence of clinical disease in the animals, and the lack of antibodies in the serum of young stock associating with them, makes untenable the otherwise logical deduction that the titer had been re-enforced by reinfection.

TABLE 3—Change in the Neutralizing Titers of 20 Cows Between the Taking of Blood Samples at 40 and 46 Months

Titers at 40 months	Titers at 46 months				
	0	1	2	3	4
0	3	2	1	....	1
1	....	....	....	....	1
2	....	....	....	1	....
3	....	....	....	....	....
4	1	....	....	5	5

The numbers in the body of the table indicate the number of animals having the respective titers at 40 and 46 months, *i.e.*, 2 cows had titer 0 at 40 months and titer 1 at 46 months.

Titers expressed as the logarithm of the l.d.$_{50}$ virus neutralized.

If there were evidence of clinical recrudescence of disease in the individual animal, the situation would be somewhat parallel to that of herpes infection in man. While the experimentally and naturally infected cattle were not examined frequently and thoroughly enough to preclude the development of a mild transitory vesiculation, the development of a disease in which virus would be shed appears unlikely in view of the freedom from antibodies of other animals in the same herds. The possibility of persisting infection can not be excluded for two reasons: First, calves born to the herds were the principal source of replacements. Because of immaturity, and the possession of passive antibody, they would have been more resistant to infection than adults[4] and, therefore, the presence of virus from a recurrent attack associated with parturition may not have been adequate to infect them. Second, it may be that virus can be shed in quantity when a suitable vector is not present to introduce it, and infection of fully susceptible residents will not be accomplished.[3] If virus was shed, suitable conditions for transmission did not occur in the period of eight years from 1949 to 1957. A few cows with high neutralization titers were still present in the herds in the spring of 1957. Replacement animals were still negative.

Whether or not virus capable of infecting other cattle may exist in animals infected at one time, certainly the persistence of the specific antibody with little decrease after four years, and in some instances for eight years, suggests that virus, in a form capable of stimulating continued antibody production, persists in the animals. The rise and fall of neutralizing titer may indicate that there is a

periodic "contained escape" and subsequent retreat of the virus to its chronic foci.

SUMMARY

Cattle usually recover from an infection with vesicular stomatitis virus within two weeks and, within a few months, may again become susceptible to inoculation. Virus-neutralizing antibodies did not disappear with the loss of refractoriness to reinfection, although the titers were sometimes reduced. The titers subsequently increased, and this fluctuation of titers of the neutralizing antibodies continued over long periods, in cycles lasting several months. Animals in herds exposed to infection during an epizootic of vesicular stomatitis in 1949 still possessed high neutralizing titers in 1952 and 1953 and, in a few animals, in 1957. Animals born in these herds after 1949 did not possess neutralizing titers in 1952 and 1953. The persistence of fluctuating titers of neutralizing antibody was studied in cattle exposed to the virus. The possible mechanism of persisting immunity is discussed.

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Experimental Mixed Infection of Cattle with Foot-and-Mouth Disease Viruses

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INVESTIGATIONS on mixed foot-and-mouth disease virus infections have not been extensive.

Vallée and Carré [15] reported dual infections in calves following inoculation with two strains of the virus, one of Vallée O and the other of Vallée A type. The presence of virus of both types was detected in the blood of about half of the infected animals. Hole [8] described dual infections in guinea pigs resulting from the inoculation of the plantar pads with O and A viruses, either mixed or separately. In both these investigations, the results of subsequent challenge demonstrated development of a bivalent immunity.

The experimental production of a dual infection of the bovine tongue with the viruses of foot-and-mouth disease and vesicular stomatitis was demonstrated by Brooksby.[1]

Several authors [5, 9, 13] have found samples of infected tongue epithelium, harvested during epizootics of foot-and-mouth disease, that gave fixation of complement with more than one type of specific serum. Whether this is due to one virus strain with a complex antigenic structure, to a strain undergoing antigenic transformation, or to a mixture of strains, is still undecided.

In several countries of South America, epizootics due to O, A, and C types of foot-and-mouth disease virus frequently occur in the same area at the same time.

The object of the experiments described in this paper was to determine whether mixed infections could be produced in cattle and to study any subsequent immunological response.

MATERIALS AND METHODS

Following the preparation and titration of virus suspensions, cattle were inoculated in the tongue with O, A, and C types. Vesicular epithelium from primary and secondary lesions was collected and examined for the presence of specific virus types and, later, serum samples were collected and examined for the presence of antibody.

Strains of Foot-and-Mouth Disease Virus.—Vallée O, Vallée A, and Waldman C type strains were selected, all isolated from epizootics of the disease in cattle in Brazil and since maintained by serial passage in cattle (table 1).

TABLE 1—Origins and Passages of Strains of Foot-and-Mouth Disease Virus Used for Inoculation of Cattle in Attempts to Produce Mixed Infections

Virus type	Origin of strain	Passage used
Vallée O	Barra Mansa, State of Rio de Janeiro; May, 1955.	2nd
Vallée A	Cruzeiro, State of São Paulo; June, 1955.	3rd
Waldmann C	Rezende, State of Rio de Janeiro; May, 1955.	4th

Inoculation of Cattle.—Six cattle, from 18 to 24 months old, were selected on the basis of the absence of foot-and-mouth disease antibodies in their serum. Multiple intradermal inoculation of the tongue was performed under chloral hydrate anesthesia. Each animal received the three virus strains in a transverse row of four sites of inoculation for each strain. Approximately 0.05 ml. of inoculum was used at each site. From the dorsal prominence to the tip of the tongue, the order of inoculation of the strains was: O, A, C, for 2 cattle; A, C, O, for 2 cattle; and C, O, A, for the remaining 2 cattle.

Preparation of Virus Suspensions.—All infective tissues were ground with sterile sand and suspended in phosphate buffer solution at pH 7.6. These suspensions were clarified by centrifugation. All dilutions are expressed in terms of the original tissue.

Titration of Virus Suspensions.—Tenfold dilutions were prepared using phosphate buffer solution. Unweaned mice, 6 to 9 days old, were inoculated intraperitoneally with 0.05 ml. of virus. Eight mice were used for each dilution and the 50 per cent end point was calculated by the method of Reed and Muench.[10]

Test of Infective Material for Specific Virus Types.—All samples of vesicular epithelium collected from the primary and secondary lesions of the inoculated cattle were titrated, and the virus type was initially determined by the complement-fixation test. Samples showing evidence of a mixed infection were then further studied by using immune, unweaned mice.

1) *Complement-fixation Test.*—The technique was essentially that described by Camargo *et al.*[3] and by Cunha *et al.*,[4] in which the percentage hemolysis is determined by spectrophotometric

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readings, using one or more units of complement, as indicated by the result of measuring the anticomplementary activity of the antigen. Minor modifications of this technique are given.

a) *Antigens.*—Antigens were prepared from a 1 in 5 tissue suspension, except in three instances, when the infected tissue weighed 0.3 Gm. or less, when 1 in 10 suspensions were prepared. Whenever the volume of the suspension was sufficiently large, two preparations were made, one by extraction with chloroform (CLE antigen) and the other by high speed centrifugation (HSC) for 15 minutes at 12,500 r.p.m. in the 40,000 r.p.m. head of a Spinco model L ultracentrifuge. When the volume of antigen was small, 0.2 ml. of each reagent was used instead of the standard 0.4-ml. volume, in which case (for spectrophotometer readings), 1.0 ml. of 0.9 per cent sodium chloride solution was added to each tube before centrifuging to deposit the erythrocytes.

b) *Antiserums.*—Hyperimmune serums, prepared in guinea pigs with standard strains of O, A, and C viruses, were used. The material from the inoculated cattle was tested using such serums.* Mouse tissue antigens were checked for virus type using serums prepared in this laboratory.

2) *Immune Unweaned Mice.*—Groups of 20 adult pregnant mice were inoculated intraperitoneally with mouse-adapted strains of O and A types. One group was given $10^{5.7}$ i.d.$_{50}$ of the thirty-second passage virus type O, and the other group was given $10^{6.3}$ i.d.$_{50}$ of the forty-ninth passage virus type A. Litters were born four to eight days after inoculation, and were used as "immune unweaned mice" when 5 to 8 days old. The possibility of utilizing the transference of maternal immunity was reported by Skinner *et al.*[12]

Examination of Cattle Serums for the Presence of Antibody.—The antibody content of the serum samples was estimated by the serum-protection test, using unweaned mice. The technique of this test and a comparison between it and the serum-neutralization test in mice has been reported.[3]

For each of the three virus types, one group of 16 mice was used for the subcutaneous inoculation of 0.1 ml. of each undiluted cattle serum sample. One hour later, four dilutions of mouse-adapted virus were inoculated intraperitoneally (0.05 ml., 4 mice per dilution). Control virus inoculations were made, using a similar group of mice which were given saline subcutaneously. The passages used of the the mouse-adapted strains were the thirty-second and thirty-third, the forty-eighth, and the fifty-fourth for the O, A, and C strains, respectively.

The protection index of a serum is the logarithmic difference between the virus titers in mice with and without serum.

RESULTS

The results of titrations in unweaned mice of the virus suspensions used for inoculation of the 6 cattle showed that they received, at each site, 10^4 i.d.$_{50}$ of virus O, $10^{5.2}$ i.d.$_{50}$ of virus A, and $10^{3.6}$ i.d.$_{50}$ of virus C.

The 6 cattle were housed in the same box

* Obtained from the Research Institute (Animal Virus Diseases), Pirbright, England.

TABLE 2—Results of Examination of Vesicular Epithelium Harvested from Cattle Inoculated with Three Types of Foot-and-Mouth Disease Virus

		Tongue epithelium				Foot epithelium				
Animal (No.)	Virus order	Time (hours)	Weight (Gm.)	Virus titer	Complement-fixation (%)	Foot	Time (hours)	Weight (Gm.)	Virus titer	Complement-fixation (%)
223	O	21	0.2	5.2	O-99	RF	72	0.2	2.8	C 91
	A	21	0.3	5.3	A-100	LF	72	0.5	5.1	C 83
	C	21	4.0	5.0	C-93	RH	72	1.0	4.4	C 45
						LH	72	0.9	3.4	Neg. ....
224	O	18	1.0	5.6	O-97	RF	72	0.6	2.7	C 49
	A	18	1.6	5.3	A-99	LF	72	0.9	4.2	C 90
	C	18	2.2	5.7	C-84	RH	48	1.0	5.5	C 77
						LH	48	0.7	5.2	C 91
221	A	18	1.3	4.5	A-93	RF	48	0.5	5.0	C 81
	C	18	2.6	4.2	C-92	LF	48	0.8	5.0	C 66
	O	18	1.7	6.2	O-54	RH	72	1.0	4.0	C 67
						LH	72	1.0	4.7	C 57
228	A	21	0.5	3.3	A-100	RF	72	1.0	3.6	C 85
	C	21	0.3	3.9	C-90	LF	72	1.1	2.8	C 85
	O	21	0.6	5.4	O-80	RH	72	0.9	4.1	C 97
						LH	72	1.3	3.6	C 88
232	C	18	1.4	6.5	C-70	RF	48	0.9	4.5	C 90
	O	18	1.6	6.4	O-91	LF	48	0.8	4.2	C 94
	A	18	2.0	5.6	A-80	RH	48	0.7	5.1	C 92
						LH	48	1.0	5.2	C 91
235	C	24	0.8	4.3	C-97	RF	72	1.1	4.7	OA–75; 61
	O	24	1.0	6.5	O-100	LF	72	1.4	6.0	OA–35; 74
	A	24	0.8	4.4	A-92	RH	72	1.3	5.6	OA–89; 96
						LH	72	0.7	5.5	OA–99; 99

RF = Right fore; LF = Left fore; RH = Right hind; LH = Left hind.

TABLE 3—Results of Examination of Vesicular Epithelium from Secondary Lesions in Which Two Virus Types Were Identified (Animal 235)

Origin of material examined	Virus titers in immune and normal mice			Results of complement-fixation tests (% fixation)			
	O-immune	A-immune	Normal	Antigen	O	A	C
RIGHT FOREFOOT							
Cattle epithelium	3.25	5.0	4.66	HSC	75	61	0
O-immune mice	.....	.....	4.40	HSC	16	100	10
				CLE	0	87	0
A-immune mice	.....	.....	3.66	HSC	80	17	0
				CLE	49	0	0
Normal mice inoculated with A-immune mouse tissue	.....	.....	6.34	HSC	100	19	0
				CLE	100	10	0
LEFT HIND FOOT							
Cattle epithelium	4.5	5.15	5.50	HSC	99	99	0
O-immune mice	.....	.....	4.20	HSC	0	100	0
				CLE	10	79	10
A-immune mice	.....	.....	4.00	HSC	92	20	0
				CLE	55	0	0
O VIRUS CONTROL 32nd mouse passage virus	<1.0	.....	5.80	HSC	100	8	7
				CLE	100	0	0
A VIRUS CONTROL 49th mouse passage virus	.....	<1.0	5.60	HSC	4	100	4
				CLE	0	100	0

HSC = antigen prepared by high speed centrifugation; CLE = antigen prepared by chloroform extraction.

stall, and their tongues were examined at 15, 18, 21, and 24 hours for the formation of primary lesions, and their feet at 48 and 72 hours for the formation of secondary lesions. Vesicular epithelium was harvested from each of the three portions of the tongue as soon as definite vesicular lesions had developed. In 3 animals, this was at 18 hours, in 2 at 21 hours, and in the remaining animal at 24 hours. Foot lesions developed on all four feet of all the cattle within 48 to 72 hours after virus inoculation. Material was harvested separately from each foot. The weight of epithelium harvested, the virus titers in unweaned mice, and the complement-fixation test results are shown (table 2).

In every case, the virus harvested from the primary lesions was of the same type as that inoculated. A triple infection was, therefore, achieved in each animal. In 5 cattle, however, the presence of only C virus was demonstrated in the epithelium of the secondary lesions. In the sixth animal, O and A viruses were present in the foot lesions. Material from the secondary lesions on two of the feet of this animal was further examined using immune unweaned mice. Serial dilutions of these foot epithelial suspensions were inoculated into unweaned mice from mothers inoculated with O virus, unweaned mice from mothers inoculated with A virus, and unweaned mice from uninoculated mothers. Serial dilutions of stock O and A viruses were also inoculated into mice of the same three groups. The results of these inoculations and of the complement-fixation test of tissue harvested from the reacting mice are given (table 3).

The complement-fixation test results with each foot epithelium sample suggest that the reaction of the O-immune mice must have been due to the A virus and, similarly, that the A-immune mice reacted to the O virus. Furthermore, the test of the virus harvested from the reacting mice showed that the heterologous virus type could be recovered from each immune group. As it was possible, therefore, to separate each virus, it can be assumed that a double infection had occurred.

The result of checking the immunity of the unweaned offspring of the inoculated female mice showed that the technique afforded protection against intraperitoneal inoculation of more than 10^4 $i.d._{50}$ (table 3).

A series of five passages was made, in normal mice, of the virus mixture isolated from the left hind foot of the cattle. The results of complement-fixation tests on the material of each passage are given (table 4). It is evident that, by the fourth passage, O virus was becoming predominant and, by the fifth passage, the A virus component of the mixture had been lost.

TABLE 4—Results of Passage in Normal Mice of the Mixture of Two Virus Types Isolated from a Secondary Lesion in Animal 235 (Left Hind Foot)

Normal mouse passage	Results of complement-fixation tests			
		Fixation (%)		
	Antigen	O	A	C
1	HSC	78	62	13
	CLE	22	22	0
2	HSC	43	70	0
	CLE	20	73	3
3	HSC	72	90	0
	CLE	20	26	0
4	HSC	100	14	6
	CLE	99	21	0
5	HSC	100	0	0
	CLE	100	4	4

HSC = antigen prepared by high speed centrifugation; CLE = antigen prepared by chloroform extraction.

Blood samples were collected from the cattle immediately before they were inoculated and at 7, 14, 21, 30, 60, and 90 days postinoculation. The serum-protection indexes obtained by testing the serums prepared from these samples are given (tables 5, 6, 7). Each of these tables includes the serum-protection indexes obtained for 2 cattle, following the inoculation of only one virus type.

All 6 cattle had high antibody levels for C virus, which were maintained throughout the experiment. Initially, the levels of antibody developed against A virus were similar to that for C virus, but this level was not maintained and it had dropped considerably after two and three months. Only 3 cattle had any significant development of O antibody, but the levels dropped rapidly and normal values were regained by the third month.

DISCUSSION

The virus of foot-and-mouth disease, after intradermal inoculation of the tongue of cattle, multiplies locally in the tongue epithelium. The virus is thereafter distributed by the blood throughout the body, and secondary lesions develop at other sites of predilection, most obviously elsewhere in the mouth and in the interdigital spaces on the feet.

The results of the described experiment show that infection with the strain of each virus type was established simultaneously at different sites on the tongue but, except in one animal, the subsequent course of the disease was apparently (by development of secondary lesions) produced exclusively by virus of one type. In the case of the sixth animal, in which O and A viruses were identified as causing the secondary lesions, it may be significant that its initial serum sample showed the highest protection index against C virus.

The fact that O and A antibodies were detected after inoculation in those cattle in which only C virus was found to cause the secondary lesions does not imply that a generalized infection was associated with these strains. The antigen produced in the local lesions might have been sufficient to stimulate this antibody response.

Skinner [11] has reported a transitory rise in heterologous type antibody to occur following infection with one type. This phenomenon may have accounted for the response observed in cerain of the cattle, e.g., in animals 223, 224, and 221 there was a small increase in O antibody that disappeared in 21 to 30 days.

The survival, following serial passage of only one component, of the mixture of strains recovered from the foot lesions of animal 235 is similar to previous findings. Hole [8] recorded that passage of two strains in guinea pigs resulted in the survival of one. Ubertini [14] found that the survival of a particular strain, on passage of a mixture of two, depended on the strains used.

In the experiment described by Vallée and Carré,[15] in which 20 calves were inoculated with O and A viruses, 8 became in-

TABLE 5—Results of Serum-Protection Tests Against Type O Virus of Serum Samples from the 6 Test Cattle and 2 Control Cattle

Animal (No.)	Serum-protection indexes shown by serum collected at intervals after virus inoculation (days)						
	0	7	14	21	30	60	90
223	0.2	0.7	1.2	1.0	0.7	0.7	0.3
224	0.0	0.0	1.1	1.9	0.0	0.3	0.0
221	0.4	1.0	2.0	1.0	0.0	0.8	0.9
228	0.4	>4.7	>5.0	4.7	1.3	0.8	0.0
232	0.7	2.2	>5.0	>4.7	3.8	2.1	0.5
235	0.4	2.0	>5.0	3.1	1.3	1.7	0.5
91 *	0.5	0.7	>5.5	>5.5	3.8	....	2.8
86 *	0.7	....	>6.0	5.3	4.7	....	1.8

* Cattle inoculated with O virus only.

TABLE 6—Results of Serum-Protection Tests Against Type A Virus of Serum Samples from the 6 Test Cattle and 2 Control Cattle

Animal (No.)	Serum-protection indexes shown by serum collected at intervals after virus inoculation (days)						
	0	7	14	21	30	60	90
223	0.0	1.0	>4.0	5.6	2.6	1.1	1.2
224	0.0	0.9	3.0	4.4	>6.0	3.5	2.0
221	0.0	4.0	>4.0	>5.7	>5.2	3.5	2.2
228	0.0	>5.7	>4.0	>5.7	>6.0	2.6	1.7
232	0.0	>5.7	>4.0	>5.7	>5.2	1.7	1.6
235	0.3	1.2	>4.0	>5.7	2.9	2.6	3.0
110 *	0.0	>4.6	>4.6	>4.6	>4.6	>4.3	>5.0
113 *	0.0	1.0	>4.6	>4.6	>4.6	>4.6	>5.0

* Cattle inoculated with A virus only.

fected with both viruses, 7 with O virus, and 5 with A virus. Henderson [6] has reported that in an experiment similar to ours, in which strains of the three virus types were inoculated simultaneously, evidence of the presence of A and C, but not of O virus, was found in the pooled blood of a group of 6 cattle.

SUMMARY

Six cattle were inoculated intradermally on the tongue with three strains of the virus of foot-and-mouth disease, the Vallée O, Vallée A, and Waldmann C types.

There was no interference in the simultaneous multiplication of these three strains in different parts of the tongue. The subsequent course of the disease was by the development of secondary lesions. In 5 of the 6 cattle, these were produced, apparently exclusively, by type C virus. In the sixth animal, O and A virus strains were identified in material from the same vesicular lesions.

All the cattle developed high levels of type C antibody subsequent to the virus inoculations. Initially, the development of type A antibody was similar, but the levels dropped more quickly. Only 3 animals showed a significant, but transitory, development of type O antibody.

TABLE 7—Results of Serum-Protection Tests Against Type C Virus of Serum Samples from the 6 Test Cattle and 2 Control Cattle

Animal (No.)	Serum-protection indexes shown by serum collected at intervals after virus inoculation (days)						
	0	7	14	21	30	60	90
223	0.5	1.3	>5.0	>5.0	>4.8	>5.3	>4.0
224	0.5	1.9	4.5	>5.0	>4.8	>5.3	>4.0
221	....	5.0	>5.0	>5.0	>4.8	>5.3	>4.0
228	1.0	>5.0	>5.0	>5.0	>4.8	>5.3	>4.0
232	1.0	>5.0	>5.0	>5.0	>4.8	>5.3	>4.0
235	1.3	>5.0	>5.0	>5.0	>4.8	>5.3	>4.3
233 *	0.5	>5.0	>4.5	>5.0	>4.8	>4.0	>4.0
236 *	0.2	>5.0	>4.5	>5.0	>4.8	>4.0	>4.0

* Cattle inoculated with C virus only.

The literature on dual infections with many viruses has been reviewed by Henle [7] and, from what is known on this subject, it is not surprising that there was no interference in the simultaneous multiplication of different strains of foot-and-mouth disease virus in different parts of the same tissue. The subsequent course of events with this particular form of multiple infection would appear to be controlled by the immune status of the animal and by the characters of the individual virus strains. The recovery of two virus strains from one vesicular lesion, as in the case of the secondary lesions of animal 235, adds more evidence to the possibility that samples of infected tongue tissue collected in natural epizootics of the disease contain strains of more than one virus type.

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Distribution of Penicillin in Mastitic Udders Following Intramammary Injection — An Autoradiographic Study

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As a routine measure, intramammary penicillin treatment for mastitis has predominated during recent years. Frequently, however, the clinical response is poor or of short duration in spite of a high sensitivity *in vitro* of the causative organism. There are no good data to show the extent to which this may be caused by inadequate distribution of the drug. Even though the milk may be free of bacteria, it is still possible that viable organisms may be present at isolated foci. MacFarlane[4] has used dyes to study the distribution of drugs introduced into the mammary gland. This method, however, suffers from the disadvantage that dyes exhibit their own distribution properties and tissue affinities.

In a study with normal udders[8] it was shown that penicillin, both as an aqueous solution and as a suspension in oil, was well distributed throughout the normal udder. In the experiments to be described here, the distribution of penicillin in diseased udders was studied and related to clinical, bacteriological, and pathological findings.

Materials and Methods

Penicillin, labeled with S^{35} and with a specific activity of 0.05 C/Gm. (Curie/gram), was injected through the teat canal into the udders of unmilked cows and goats with various types of mastitis.

The mastitis was detected by clinical examination. Before the injection of penicillin, milk samples were taken and examined by routine bacteriological methods. Leukocyte counts were carried out by the method of Prescott and Breed.[5] The penicillin was administered either as an aqueous solution or as a suspension in oil. For cows, 6 ml. of vehicle per quarter was used, and for goats, 3 ml. per half udder. The penicillin concentration was 1 mg. per milliliter, corresponding to a radioactivity of approximately 50 μc/ml.

Eight to 15 hours after injection, the animals were killed and the udders frozen in acetone-carbon dioxide ice (−70 C.) and then sliced into 50-μ sections. The sections were dried and pressed against x-ray film. Sectioning, drying, and exposure of the sections took place at −10 C.

The technique for sectioning and determining the distribution of penicillin in normal goat udders by autoradiography has been described.[6] The sectioning technique has been modified for bovine udders so that only one quarter appears in each section rather than the whole udder as described for goats.

A detailed examination of the pathological changes has been carried out on samples removed from various portions of the cut surface of the udder block. The samples were taken from areas of special interest for the study of penicillin distribution as determined from a preliminary autoradiograph.

Results

Using diseased cow and goat udders, the distribution of penicillin was found to be irregular, with sections of varying size showing a complete absence of penicillin.

The distribution varied considerably among the autoradiographs. Some showed a uniform distribution throughout the udder (fig. 1A and 4A). However, a comparison of autoradiographs and tissue sections of some regions showed a low concentration or a complete absence of penicillin. Such areas extended over single lobules or were the size of a hen's egg (fig. 4).

In other diseased udders, a poor distribution of penicillin was obtained. Some autoradiographs showed penicillin only in the larger milk ducts (fig. 2) or in the ducts and limited portions of the parenchyma (fig. 3). In one udder, penicillin was found only in the teat cistern (fig. 7).

The vehicle used did not apparently affect the general distribution of the penicil-

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The authors thank Dr. E. Hanko, State Veterinary Medical Institute, Stockholm, for his assistance with the pathological investigations.

* The corresponding American titles would be D.V.M., Ph.D.

† The corresponding American title would be D.V.M.

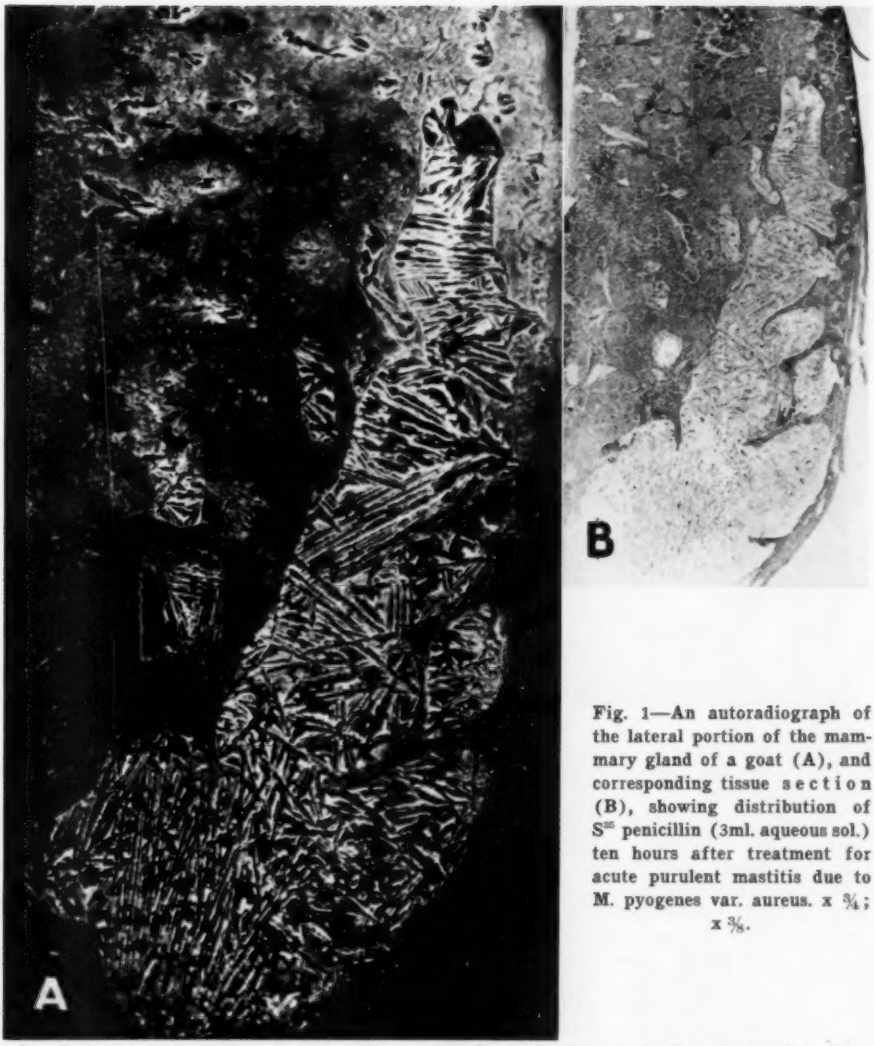


Fig. 1—An autoradiograph of the lateral portion of the mammary gland of a goat (A), and corresponding tissue section (B), showing distribution of S^{35} penicillin (3ml. aqueous sol.) ten hours after treatment for acute purulent mastitis due to M. pyogenes var. aureus. x ¾; x ⅜.

Distribution in the parenchyma is fairly uniform except for smaller amounts in the central and medial portions. Some lobules contain only a little or no penicillin. It is present in the intralobular tissue and the interlobular interstitium bordering on the lobules in most parts of the gland. The gland was somewhat swollen, warm, and firm but the milk appeared normal (sediment 0.3 vol. %; 30 x 10^6 leukocytes/ml.). The edema, inflammatory products and desquamated epithelial cells in the ducts and in many alveoli of the gland affected the distribution of penicillin.

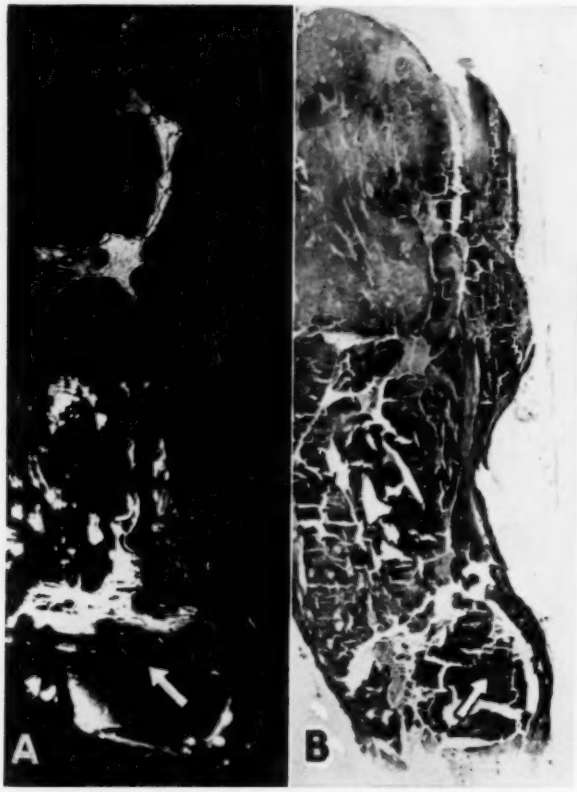


Fig. 2 — Autoradiograph of an actively lactating mammary gland of a goat (A), and corresponding tissue section (B) showing distribution of S^{35} penicillin (2 ml. in oil) eight hours after treatment for acute necrotic mastitis due to coliform infection. x ¾.

A large amount of penicillin is in some of the largest milk ducts, smaller amounts are in the surrounding tissue, with none in the remainder of the gland. The gland contained a watery secretion with some blood clots (arrows) which were an additional factor in affecting the distribution of penicillin.

lin. Both aqueous solutions and suspensions in oil showed approximately the same degree of irregularity in the autoradiographic pictures as a result of the penicillin being prevented from penetrating into certain portions of the tissues because of the pathological changes.

The diseased udders used in this study, as well as as the normal udders previously investigated, show the same general pattern of penicillin distribution, *i.e.*, a relatively higher concentration of penicillin in oil in the milk ducts and a lower concentration in the parenchyma, as well as a certain tendency for penicillin in oil to collect dorsally in the udders.

A lack of medium (milk) in which the penicillin can be dispersed seems to be one cause of incomplete distribution. Extremely inadequate distribution is shown (fig. 5 and 6) in the dorsal portions of quarters which had been treated immediately after lactation ceased. The pathological changes in these instances were such that they alone could probably not account for the poor distribution.

Study of the autoradiographs has shown that distribution of penicillin is essentially dependent upon satisfactory passage through the ducts and that spread by means of diffusion through tissue occurs only to a limited extent. Pathological changes in mastitis can influence passage through the ducts so that diseased as well as normal tissue in more dorsal portions of the gland are not exposed to penicillin administered through the teat canal.

Figure 1 illustrates a case in which edema and changes in the milk, not noticeable macroscopically, affected distribution to only a slight degree. On the other hand, in the gland shown in figure 2, where pronounced changes in the milk, and necrosis were present, the penicillin was distributed inadequately.

In chronic mastitis, proliferation of epithelial cells which more or less fill the lumens of the alveoli and ducts is a commonly encountered lesion.[1] The penicillin-free areas, as in figure 4, are often the sites of more or less pronounced fibrous tissue proliferation. In the udder shown in figure 7, in which the penicillin could be found only in the teat cisterns, granulation tissue had completely obstructed the milk ducts.

In the investigation of distribution within healthy udders, penicillin was found in the ducts and in the parenchyma, while none, or only insignificant amounts, could be found in the interstitial tissues. In mastitic udders, the major portion of the penicillin was definitely situated in the milk ducts and, to various degrees, in the parenchyma, although some had also penetrated into the interstitial tissues in several places. The latter was especially marked in portions in which edema and marked infiltration of leukocytes indicate an acute inflammatory process, however, penicillin penetrated slightly, or not at all, into the interstitial tissue of areas in which acute inflammation had subsided and the process had taken on a fibrous character, and areas where the normal mammary tissue had remained unchanged.

DISCUSSION

The distribution of penicillin within the udder in the early stages of acute mastitis, before demonstrable changes in the milk have appeared, has, in general, been good in spite of pronounced edema and the presence of inflammatory changes in the tissue. In a few lobules, penicillin was present only in insignificant or nondetectable amounts.

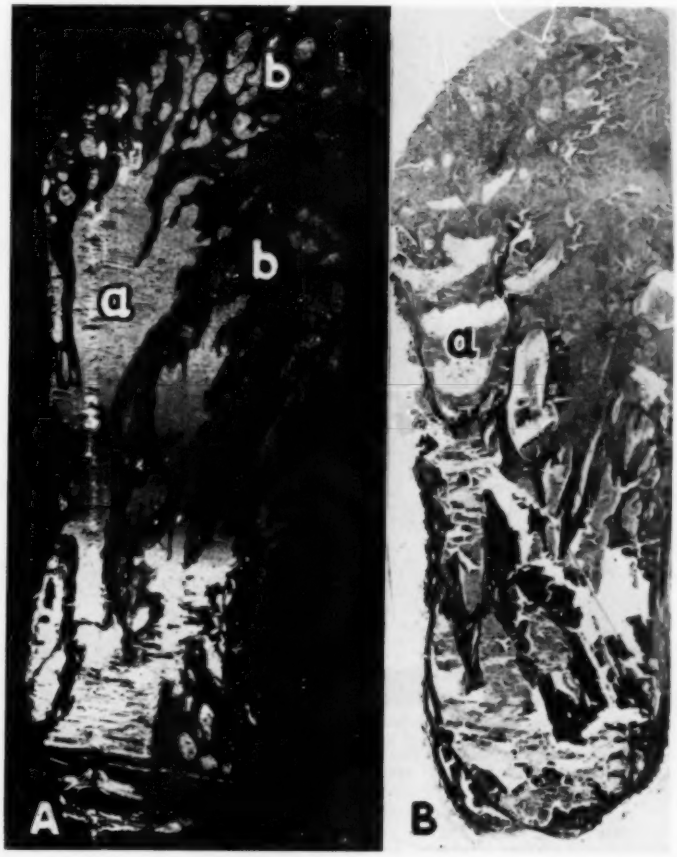


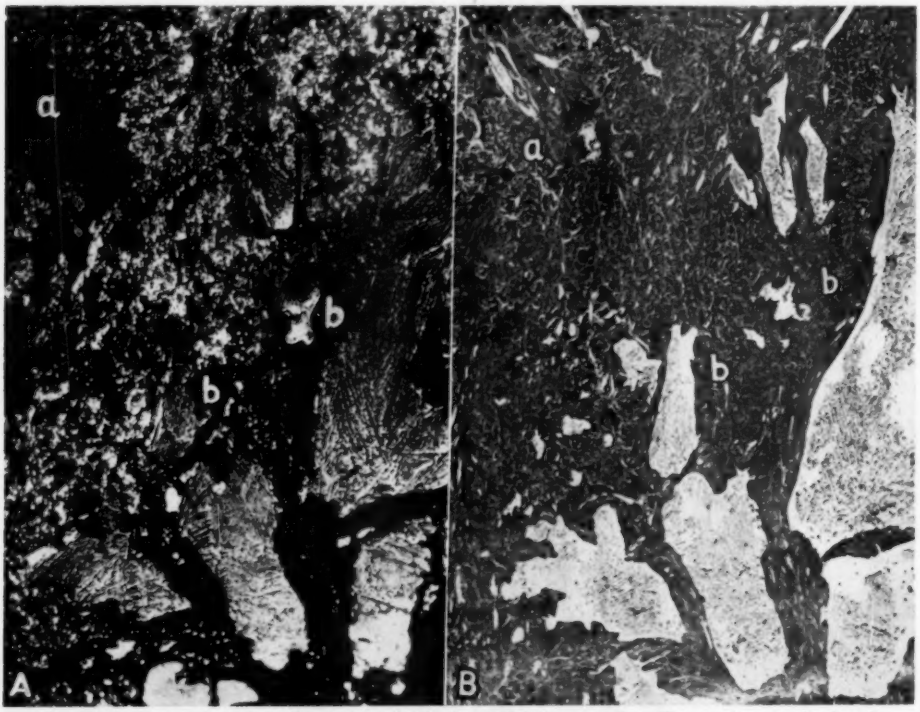
Fig. 3 — Autoradiograph of an actively lactating udder of a goat (A), and corresponding tissue section (B), showing distribution of S^{35} penicillin (3 ml. in oil) eight hours after treatment for acute purulent mastitis with chronic inflammatory changes. x 1.

Gram-negative rods resembling Pasteurella were isolated in pure culture. The udder contained a watery, bloody secretion with small clots. Irregular, large portions of the gland do not contain penicillin. Much penicillin is in the large, dilated milk ducts (a) and some is in the interstitial tissues bordering on them. Lobules with moderate or slight amounts of penicillin (b) alternate with lobules without penicillin.

When penicillin was introduced into udders with acute mastitis, in which changes in the milk could be demonstrated, the distribution was unsatisfactory and penicillin could be demonstrated only in the larger milk ducts and in a few lobules. In these cases, desquamated epithelial cells, inflammatory products, blood clots, necrosis, and edema influenced the distribution to such a degree that antibiotic treatment through the teat canal would seem to be of dubious value.

In older mastitis, with no, or only slight changes visible in the milk samples, the distribution of penicillin was good through the whole udder, although areas of varying sizes showed only slight or undetectable amounts of penicillin. The more important factors contributing toward disturbances in distribution seem to be the presence of desquamated epithelial cells and inflammatory products within the alveoli and ducts in some portions, and proliferation of epithelial cells and interstitial connective tissue. Within the blocked portions, it is possible to find active inflammatory processes, and even normal tissue. Blockage of secondary milk ducts can thus prevent penicillin from reaching areas that are still normal.

In mastitis with a prolonged course, such as some chronic streptococcic infections in which only small areas at a time are involved in acute inflammatory processes, there are grounds for assuming that the results of intramammary antibiotic treatment will be relatively good provided that widespread induration is not present. If large portions of the mammary parenchyma are simultaneously involved in acute in-



The milk appeared normal (sediment 0.1 vol. %; 2 x 10^6 leukocytes/ml.). The quarter was hard and nodular. The distribution of penicillin is fairly uniform. Some smaller areas and single lobules contain small amounts, while other similar areas, especially where connective tissue proliferation is most pronounced, contain none. A detectable amount is present in some interstitial tissue. An indurated area without penicillin (a) and parenchyma without penicillin (b) are shown.

Fig. 4—Part of an autoradiograph of an actively lactating udder of a cow (A), and corresponding tissue section (B), showing distribution of S^{35} penicillin (6 ml. in oil) 15 hours after treatment for chronic purulent mastitis due to Str. dysgalactiae. x ½.

Firm areas in the ventral part of the udder contained a water-like secretion with small clots (sediment 13 vol. %; 100 x 10^6 leukocytes/ml.). Both lactating and nonlactating lobules are present. A cross section (a) of a large milk duct is shown. Except for a small area in the dorsal part, penicillin is present only in the ventral third of the gland. Much is present in the cistern and the larger milk ducts, a little is fairly uniformly distributed in the parenchyma, and moderate to small amounts are scattered in the interstitial tissue.

Fig. 5—Autoradiograph of a nonlactating quarter of a cow (A), and corresponding tissue section (B), showing distribution of S^{35} penicillin (6 ml. aqueous) 15 hours after treatment for chronic purulent mastitis due to Str. agalactiae. x 2/3; x ½.

Fig. 6—Autoradiograph of a nonlactating quarter of a cow (A), x ½; and corresponding tissue section (B), x 2/5; showing distribution of S^{35} penicillin (6 ml. aqueous) 15 hours after treatment for chronic purulent mastitis due to Str. agalactiae. Fig. C and D are enlargements (x 3/2) of portions of Fig. A and B to show presence of penicillin in the interstitium (above "c").

Distinct indurated portions are present throughout the quarter. The milk was somewhat yellowish (sediment 0.2 vol. %; 1×10^6 leukocytes/ml.). Penicillin is present only in some of the larger milk ducts and in the surrounding parenchyma and interstitium. A large milk duct (a) and the udder cistern (b) are indicated. Figures 6C and D are enlargements of 6A and B to show details. x ¾.

flammatory processes, the possibility of producing sterile conditions within the udder is not at all promising.

The presence of only a small volume of medium (milk) in which the penicillin can be dispersed seems to be a factor which reduces the efficiency of distribution. This may indicate that the amount of vehicle used with penicillin ought to be increased in order to achieve better distribution when treating mastitis during the dry period. The amount of vehicle used in lactating udders does not seem to have any significance.

The observation that penicillin has a tendency to penetrate into the interstitial tissues in areas involved in acute inflammatory processes has already been mentioned. Previous investigations have demonstrated that, under the influence of acute inflammatory processes, penicillin in a higher degree

can pass from blood to milk.[1, 6] Similar observations have also been made concerning the barrier between blood and spinal fluid in cases of meningitis.[3]

A single intramammary treatment for mastitis usually gives an unsatisfactory result. In the case of repeated intramammary treatments, it can be postulated that the first treatment affects the most easily available inflamed areas and that suitable conditions are created for the deeper penetration of penicillin upon further administration. This raises the question of the degree to which mastitis with obstructing lesions, such as those formed by proliferation of epithelial cells, is amenable to this form of treatment.

As tissue debris blocks the dissemination of therapeutic agents through the ducts, proteolytic enzymes introduced simultaneously with a drug might contribute toward more efficient distribution. This possibility ought to be investigated more closely.

Theoretically, the most effective therapeutic method for the treatment of mastitis seems to be systemic administration of antibiotics. Earlier autoradiographic investigations of the distribution of penicillin in small laboratory animals have shown that high concentrations can be obtained in the mammary glands.[7] Penicillin administered intravenously has been shown to penetrate even relatively thick capsules of experimentally produced abscesses.[7] Several in-

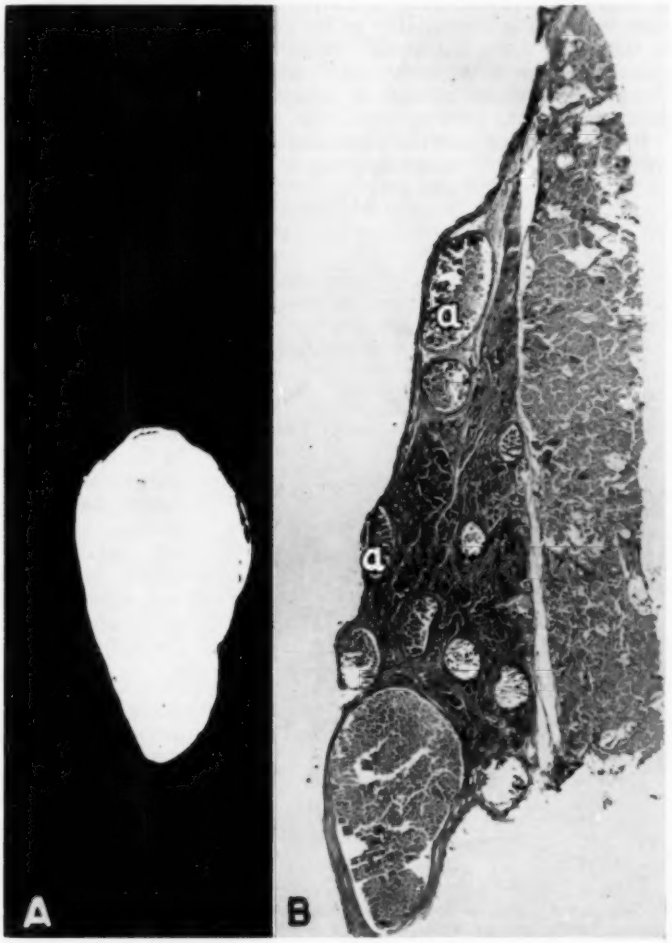


Fig. 7—Autoradiograph (A) and corresponding tissue section (B), ten hours after administration of S^{35} penicillin in 3 ml. aqueous solution, showing complete lack of distribution from the teat cistern in a severe case of chronic mastitis due to M. Pyogenes var. aureus. The teat cistern, open space in figure A, is shown at the lower left in Figure B. The markedly atrophic gland contained a thick greenish-yellow secretion and several abcesses (a). x ¾.

vestigations carried out on cattle have shown that systemic treatment with larger doses of antibiotics can produce therapeutic concentrations in the milk.[2] General adoption of this therapeutic method is, however, considered to be rather expensive at present.

SUMMARY AND CONCLUSIONS

The distribution of penicillin in mastitic udders following intramammary injection was studied using S^{35}-labeled penicillin and autoradiography.

In the diseased udders investigated, the drug was usually unevenly distributed, with regions of varying sizes showing little or none. Administration either as an oil or aqueous solution gave essentially the same result. The accessibility of the tissues to the drug depended mainly upon clear passage in the milk ducts, with diffusion through the tissues being of minor importance.

It is postulated that the failure of penicillin treatment of mastitis often results from inability of the drug to reach the diseased areas.

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The Use of Huddleson's Brucella M Vaccine Under Field Conditions

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THE PREVENTION of brucellosis in cattle has challenged the minds of investigators for a half century or more. Its eventual eradication depends upon the effectiveness of preventive methods as well as the removal of the infected animals.

A suitable immunizing agent for brucellosis should meet the following requisites: (*a*) It should not produce a progressive type of disease or establish a carrier state in the species of animal in which it is injected; (*b*) the agent should not cause the production of specific serum agglutinins that persist for more than a few months and, thus, increase the difficulty of distinguishing, by serological test, infected animals from those not infected; (*c*) it must engender sufficient resistance in susceptible animals to prevent the spread of the disease in herds while the infected animals are being eliminated, and to prevent the introduction of the disease in disease-free herds.

Despite the fact that *Brucella abortus* strain 19 has been shown by controlled laboratory and field experiments to confer a useful degree of resistance to brucellosis in cattle, its use stimulates the production of agglutinins which may persist at a diagnostic level for years. This persistence of titer interferes with the disease control program because there is no good practical method for differentiating the vaccinal from infection titers. Because of this and other disadvantages a continuing search has been made for improved Brucella vaccines. One of these recently advanced as an improved vaccine has been Huddleson's mucoid (M) vaccine.[8]

Ohio investigators[4-7] were the first to study Brucella M vaccine under well-controlled conditions. They reported that while Brucella M vaccine did not produce persistent blood-agglutinin titers, its use did not seem to produce as good resistance as did strain 19 vaccine. These results were later confirmed by Bryan *et al.*[3] and Berman *et al.*[2]

The above results were obtained when the experimental cattle were exposed to an artificial challenge dosage of 7.5×10^5 to 14×10^6 virulent organisms. Since the true dosage and virulence of field exposure were not known, it was thought that the resistance engendered by Brucella M vaccine might make it a satisfactory preventive treatment under field conditions. It was with this objective in mind that the following work was initiated.

MATERIALS AND METHODS

In these field studies, 1 ml. of the vaccine * was injected subcutaneously at the upper portion of the area immediately posterior to the scapula.

The blood serum-agglutination test was conducted by the usual tube method. Serum in two-fold dilutions ranging from 1:25 to 1:12,800 and a standardized BAI antigen were used. The tests were read following incubation at 37 C. for 48 hours.

Whenever possible, aborted fetuses were obtained from the vaccinated and control animals that aborted and were examined for the causative agent. Milk samples from animals in certain herds

TABLE 1—The Classification of Brucella M-Vaccinated Animals at the Termination of the Test

Herd (No.)	Adults			Calves		
	Neg.	Susp.	Pos.	Neg.	Susp.	Pos.
1	26	5	8	4	1	3
2	67	4	6	17	0	0
3	6	0	4	0	0	0
4	0	2	12	3	0	1
5	51	9	0	4	1	0
6	10	3	4	2	0	0
7	24	1	15	1	2	5
8	5	0	6	0	0	1
9	10	2	9	0	0	0
10	12	0	3	2	0	0
11	17	3	5	3	0	2
12	24	3	18	0	0	0
	252	32	90	36	4	12
Removed during exper.	35	8	12	6	0	0
Totals	287	40	102	42	4	12
Grand total—487						

From the Department of Veterinary Science, Ohio Agricultural Experiment Station, Wooster. From a dissertation submitted to the Graduate School, Ohio State University, Columbus, in partial fulfillment of the requirements for the Ph.D. degree.

The senior author is now on a special mission in South America for the Food and Agriculture Organization of the United Nations.

* The vaccine was supplied through the courtesy of Dr. I, Forest Huddleson, from current production lots of the Brucella Laboratory, East Lansing, Mich.

were examined at different periods for the presence of the vaccine strain and the causative microorganism. Attempted recovery of Brucella organisms was limited to the inoculation of Tryptose agar plates with milk, stomach contents of the aborted fetus and, when possible, vaginal swabs taken from the recently aborted dam. Duplicate plates were inoculated with each material. One of each set of plates was incubated under increased CO_2 tension. Following a minimum of four days of incubation at 37 C., colonies showing Brucella characteristics were selected for further identification. Saline suspensions of the selected cultures were used as the antigen in agglutination tests with known positive and negative *Br. abortus* serums. Huddleson's dye-plate method was used for species determinations.

The Brucella M vaccine was used in 12 Ohio dairy herds. At the beginning of the experiment, Brucella-infected animals were present in all 12 herds. The infection had been diagnosed from three to 18 months prior to the use of the vaccine.

All of the 487 cows and heifers vaccinated with Brucella M vaccine at the beginning of the test were negative to the blood-agglutination test at the time of vaccination. Sixty cows, negative to the agglutination test, were retained as unvaccinated controls.

The chief criterion used to determine the absence or presence of brucellosis in the animals before and after the injection of vaccine was the blood serum-agglutination titer of each animal. Blood samples were taken from all the animals and tested on the day of, or a short time before, vaccination and at intervals of three to four months during the two-year period of observation. In certain herds, the animals were bled and tested at weekly or monthly intervals after injection in order to observe the rise and fall in the blood-agglutination titer.

RESULTS

Some of the adult Brucella M-vaccinated animals exhibited a 1 to 2 dilution rise in blood titer approximately ten to 15 days following the injection of the vaccine. In all instances, the blood titers declined to a negative level within 90 days after vaccination except in those animals that became permanently infected with the virulent organisms.

The vaccination of some lactating animals resulted in some reductions of milk flow, but no serious postvaccinal reactions were seen.

No trend regarding the effect of the use of Brucella M vaccine on reproduction was observed. No therapeutic value from the use of the vaccine on infected animals was demonstrated.

The classification at the termination of the tests, as determined by the blood-agglutination test, of all Brucella M-vaccinated animals is shown (table 1).

Of the 429 Brucella M-vaccinated adults, 102 exhibited positive agglutination titers at the end of the two-year period of observation, 40 showed suspicious reactions, and 287 remained negative. Twelve of the calfhood-vaccinated animals became positive to the blood test and 42 remained negative.

During this same period, there were 45 abortions, eight premature calvings, and 490 normal calvings from adult vaccinated cows. Seven heifers aborted and 24 calved normally (table 2).

TABLE 2—Calvings During the Test Period

Herd (No.)	Adults			Heifers		
	Normal	Premature	Aborted	Normal	Premature	Aborted
1	56	5	1	4	0	3
2	86	0	2	6	0	1
3	14	0	2	0	0	0
4	14	0	5	4	0	0
5	46	0	1	2	0	0
6	29	0	2	0	0	0
7	57	0	9	1	0	3
8	12	1	6	0	0	0
9	35	1	2	0	0	0
10	22	0	2	2	0	0
11	42	0	1	5	0	0
12	77	1	12	0	0	0
	490	8	45	24	0	7

In all herds except one, approximately 10 per cent of the negative adult animals were left as unvaccinated controls. They were kept with the permanently infected and with the Brucella M-vaccinated animals, no attempt being made to segregate any of the groups.

All of the 60 unvaccinated controls were negative to the blood-agglutination test when the test began. During the two-year period of observation, 21 cows and heifers became positive, 9 became suspicious, and 30 remained negative to the blood-agglutination test. During this same period, 11 aborted, 3 calved prematurely, and 37 calved normally (table 3).

Approximately 36.9 per cent of the controls became infected, according to the blood-agglutination titers while, under the same exposure, 23.4 per cent of the Brucella M-vaccinated animals exhibited reactor titers (table 4). These differences were more marked when the calvings of these two groups were considered. Approximately 27.4 per cent of the controls

TABLE 3—Agglutination-Negative Nonvaccinated Controls

Herd No.	Total No. controls	Titer change: Adults Neg.	Adults Susp.	Adults Pos.	Heifers Neg.	Heifers Susp.	Heifers Pos.	Calvings: Normal	Calvings: Premature	Calvings: Abortions
1	(10)	4	3	3	0	0	0	0	1	4
2	(0)	0	0	0	0	0	0	0	0	0
3	(5)	4	0	1	0	0	0	5	0	0
4	(2)	....	....	....	1	0	1	2	0	0
5	(9)	8	1	0	0	0	0	8	0	0
6	(4)	4	0	0	0	0	0	3	0	0
7	(8)	2	3	3	0	0	0	3	0	0
8	(3)	1	0	2	0	0	0	0	0	2
9	(3)	0	0	3	0	0	0	0	0	0
10	(3)	2	1	0	0	0	0	2	0	0
11	(6)	3	1	2	0	0	0	7	0	0
12	(7)	1	0	6	0	0	0	7	2	5
Totals	60	29	9	20	1	0	1	37	3	11

aborted or had premature calves while only 8.8 per cent of the Brucella M-vaccinated animals aborted or had premature calves.

DISCUSSION

This experiment was designed primarily to evaluate the resistance engendered in cattle by Brucella M vaccine when challenged by maintaining them in herds where brucellosis was known to exist.

Under the conditions of this experiment, the data show that the use of Brucella M vaccine did produce a certain degree of resistance to a field exposure of virulent Brucella organisms. Statistical analysis, by the *chi*-square test, of the incidence of reactor titers in the Brucella M-vaccinated animals showed them to be significantly different from the incidence of reactors in the nonvaccinated controls. While it is true that there was a significant difference at the 1 per cent level in the incidence of abortion between the two groups, nevertheless, since the causative agent associated with the abortions was determined in only a few instances, these observations were not used as a criterion for evaluating the vaccine.

Several important factors should be considered in evaluating the Brucella M vaccine under the conditions of this experiment. First of all, the number of nonvaccinated controls was not as large as was desired. They were selected largely by the owners and many were not valuable animals, often because of poor breeding performances. The status of animals at the time of vaccination should also be considered in the evaluation of this vaccine. It has been demonstrated by Bendixen and Blom [1] that more than 200 days may elapse before cattle show a significant agglutination titer after a known exposure to *Br. abortus*. Therefore, one blood test does not insure that all animals negative to the test were free from the infection at the time of vaccination. Ordinarily, a sizeable number of controls would tend to minimize the importance of this source of error.

The results seem to confirm those obtained in our control laboratory studies of Brucella M vaccine.[4-7] In these trials, it was concluded that, although Brucella M vaccine did not offer the protection afforded by the use of strain 19 vaccine, nevertheless, Brucella M vaccine was not entirely devoid of value as an immunizing agent against bovine brucellosis.

The vaccine did not cause blood serum agglutinins to develop for an S-phase antigen in a titer higher than 1:100 dilution.

TABLE 4—A Comparison of the Brucella M Vaccinated Animals and the Controls at the Termination of the Experiment

	Total No. in exper.	Titers at termination of exper.: Remained negative	Suspicious (%)	Positive (%)	Total Calvings	Calvings: Normal (%)	Premature (%)	Aborted (%)
Brucella M vaccinates	487	67.5%	9.1	23.4	561	91.2	1.4	7.4
Controls	60	52.6%	10.5	36.9	51	72.6	5.9	21.5

As a rule, specific agglutinins in the titer of 1:25 could not be demonstrated in the serum of the animals 90 days after injection of the vaccine.

SUMMARY

Brucella M vaccine was injected subcutaneously into 487 serologically negative cows and heifers located in 12 privately owned dairy herds to determine its capability of engendering an active immunity against brucellosis.

Each of the 12 herds contained animals showing serological evidence of brucellosis and, in most of them, infected animals had been present only a short time before vaccine administration. In 11 of the 12 herds, blood-negative, nonvaccinated animals were left to serve as controls.

Of the 487 vaccinated animals, 114 (23.4%) became reactors during the 24-month observation period. During the same period, 21 (36.9%) of the 60 nonvaccinated controls became reactors. Approximately 27.4 per cent of the control animals aborted or had premature calves while only 8.8 per cent of the vaccinated animals aborted or had premature calves.

Some of the Brucella M-vaccinated animals exhibited a one- or two-dilution rise in blood titers approximately ten to 15 days following the injection of the vaccine. In all instances, the blood titers declined to a negative level within 90 days after vaccination except in those animals that became permanently infected with the virulent organisms.

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The Effects of Prepartum Administration of Progesterone to the Cow

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RETENTION of the fetal membranes in the cow was experimentally produced by removal of the corpus luteum of pregnancy at the Wisconsin Station by McDonald *et al.*[5] and the study was extended by Venable and McDonald [8] at the Oklahoma Station. Histological study of the fetal placentas from a limited number of these animals indicated that the retention was of the noninfectious type.

Not all field cases of retention of fetal membranes can be accounted for on the basis of infectious agents.[2] The retention caused by corpus luteum removal [5] was prevented by injections of progesterone prior to parturition. As a result of this work, it became apparent that a study should be made to determine if exogenous progesterone would reduce the incidence of retention of the placental membranes under field conditions.

MATERIALS AND METHODS

The 186 cows and heifers of five breeds, in the University of Illinois dairy herd, were allotted at random to two groups. The treated animals were given intramuscular injections of 500 mg. of microcrystalline progesterone * on the thirty-third, twenty-third, and thirteenth days, and 100 mg. of progesterone in sesame oil on the third and second days prior to the expected day of parturition for cows of the breed.[1] A record was kept of the sex of each calf, the length of gestation, the duration of retention of the fetal membranes and, where possible, a biopsy of the maternal-fetal placental attachment was taken soon after parturition. The lactation which followed the series of progesterone injections was compared with the preceding and succeeding lactations of the cows and the succeeding lactations in the heifers to determine if the injections affected production. During the course of the injections, a 15-day record of rectal temperatures was made on 5 treated cows, 6 control pregnant cows, and 4 nonpregnant cows to determine if the temperature was elevated by the injections, as had been reported in the human being.[1]

RESULTS

Three cows given injections had twins and all 3 expelled the fetal membranes normally. In the control group, 5 cows had twins and 4 of them retained their fetal membranes. The incidence of retention of the fetal membranes in the animals which had single calves is indicated (table 1). There is no significant difference in the incidence of retention of the fetal membranes between the two groups. No significant alteration of gestation length (excluding twins) resulted from the progesterone injections when all breeds were considered (table 2). The injections likewise caused no differences in rectal temperatures, since cows in the injected group, the control group, and the nonpregnant group had mean rectal temperatures, respectively, of 101.81, 101.74, and 101.80 F. A comparison of the post-treatment annual milk production record with the cow's own succeeding or preceding production record revealed no significant difference in either the injected or control group.

TABLE 1—The Effect of Progesterone on Fetal Membrane Retention

	Heifers			Cows			Total		
	Retained		Expelled	Retained		Expelled	Retained		Expelled
Group	(No.)	(%)	(No.)	(No.)	(%)	(No.)	(No.)	(%)	(No.)
Treated	3	9.4	29	3	5.3	53	6	6.1	82
Control	1	3.0	32	6	10.5	51	7	7.8	83

From the Department of Veterinary Physiology and Pharmacology and Illinois Agricultural Experiment Station (McDonald), and the Department of Dairy Science and Illinois Agricultural Experiment Station (Hays), University of Illinois, Urbana.

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TABLE 2—The Effect of Progesterone on Gestation Length *

Breed		Cows				Heifers				Total	
		Male		Female		Male		Female			
		Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control
Ayrshire	No.	5	3	5	8	4	3	4	4	18	18
	Mean	+1.8	+4.0	+2.4	+1.6	+2.8	+4.3	−0.2	+1.0	+1.7	+2.3
Brown Swiss	No.	6	4	3	6	2	4	3	1	14	15
	Mean	+1.8	−0.2	−1.0	−0.8	−0.5	−1.2	−1.7	+4.0	+0.1	−0.4
Guernsey	No.	3	3	1	2	1	1	2	....	7	6
	Mean	+6.3	+6.7	+1.0	+2.0	+5.0	+8.0	−2.0	....	+3.0	+5.4
Holstein	No.	9	11	10	10	6	8	7	5	32	34
	Mean	+2.7	+3.1	−0.3	+2.7	+2.3	+2.1	−0.4	+3.2	+1.0	+2.8
Jersey	No.	4	4	10	3	2	1	1	1	17	9
	Mean	+5.5	+6.5	+3.0	+7.3	+5.0	+2.0	+2.0	−1.0	+3.8	+5.4
Total	No.	27	25	29	29	15	17	17	11	88	82
	Mean	+3.1	+3.7	+1.3	+2.1	+2.6	+2.0	−0.6	+2.1	+1.7	+2.6

* Calculated as deviation in days from the average for the breed: Ayrshire, 279; Brown Swiss, 290; Guernsey, 283; Holstein, 279; Jersey, 279.[2]

DISCUSSION

The progesterone administration apparently did not noticeably affect the treated cows. It can be postulated that the circulating level of progesterone in the control cows was sufficient to meet the threshold required to prevent the retention of the fetal membranes. Progesterone was not expected to affect lactation, since the mammary gland was already developed when the injections were started. The dose of progesterone administered to these animals does not appear to be high enough, or the same mechanism does not exist in the cow as in the human being whereby progesterone elevated body temperature.

The data regarding length of gestation is of interest, since the addition of this dosage of progesterone did not suppress the forces favoring parturition in the cow as has been demonstrated in the rabbit [3] and rat.[6]

The dosage is of concern in every pharmacological study of this type, but the dosage of exogenous progesterone used is that which was established by McDonald *et al.*[4] as a quantity which could effectively replace luteal function in the cow, as measured by prevention of abortion after removal of the corpus luteum. However, abortion is only one criterion of progesterone deficiency, and perhaps the threshold for progesterone is different for other physiological responses such as lactation, influence on gestation, and body temperature effect.

SUMMARY

An experiment involving 186 dairy cows was performed to determine if progesterone administration for approximately one month prior to parturition would affect the expulsion of the fetal membranes, the length of gestation, the subsequent lactation, the body temperature, and other physiological mechanisms of the cows. There were no significant differences between the treated and control groups, as measured by these criteria. The progesterone apparently produced no untoward effects in these animals.

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Morphological and Serological Characteristics of Fresh Isolates of *Vibrio Fetus* and Other Vibrios Inhabiting the Bovine and Ovine Genital Tracts

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VIBRIOS which culturally resemble *Vibrio fetus* have been isolated from vaginal and cervical mucosa, preputial washings, and semen of cattle and sheep. These strains were distinguished from *V. fetus* by Bryner and Frank [2] as being producers of hydrogen sulfide and nonproducers of catalase, while Kuzdas and Morse [8] demonstrated the ability of these strains to propagate in mediums containing 3.5 per cent sodium chloride. Such strains have often been designated as "saprophytic."

In a previous paper, Ristic *et al.*[11] reported the existence of one smooth and four distinct nonsmooth colonial types with correlated cell morphology when *V. fetus* stock cultures were examined by oblique, indirect, transmitted light. In addition, antigenic heterogeneity among *V. fetus* variants was demonstrated by cross-agglutination and agglutinin-absorption techniques.[11,13] Considering the long periods of *in vitro* maintenance of these cultures, it was reasonable to assume that the variants described may have represented secondary mutants from nonsmooth forms, rather than first-step variants from the normal, smooth form. Thus, further studies on freshly isolated strains were needed to determine the state of the organism *in vivo* and the deviation from the parent type in successive generations *in vitro*, with reference to colonial form and cellular and antigenic properties.

The present report describes: (1) colonial types and cellular morphology of freshly isolated *V. fetus* and saprophytic Vibrio strains inhabiting the bovine and ovine genital tracts; (2) dissociation of these cultures in eight different mediums generally used for maintenance of *V. fetus* stock cultures; and (3) antigenic characteristics of certain variants, with reference to serological activity with antiserums secured from immunized rabbits and naturally infected cattle.

MATERIALS AND METHODS

Vibrio fetus and other Vibrio strains used in this study were isolated from one of the following sources: stomach contents of aborted fetuses, vaginal, cervical, or preputial mucosa, or semen of cattle and sheep. The cultures were designated "freshly" isolated, since they were studied as original isolations or subcultures of strains lyophilized upon original isolation. The original cultures and subcultures from lyophilized strains were started and maintained in these studies by propagation on Brucella * agar plates, containing 2 per cent agar. After incubation for 72 hours at 37 C., in an atmosphere of 10 per cent CO_2, the colonies were examined under a 10 to 45 x magnification, using oblique, transmitted light, according to the method of Henry.[5] The colonial type observed, with concomitant cellular morphology, was assumed to be that existing *in vivo*.

Colonies were suspended in a small amount of saline solution, and the density adjusted to approximate the tube two McFarland standard. The suspension (0.2 ml.) was inoculated into tubes of each of the following mediums: Brucella semisolid broth, tryptose semisolid broth, and thioglycolate semisolid broth, each containing 0.16 per cent agar; Brucella semisolid broth containing 0.16 per cent agar and 0.05 per cent cystine; cystine trypticase semisolid broth ** with 0.35 per cent agar; blood agar slant containing tryptose blood agar base with 5.0 per cent citrated blood; Brucella agar slant containing 2.0 per cent agar and 0.05 per cent cystine; and Brucella agar slant with 2.0 per cent agar. The cultures were incubated in the manner described above. After incubation, the cultures were preserved at room temperature for one week, at which time subcultures were made on the same mediums, using

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** Baltimore Biological Laboratories, Baltimore, Md.

TABLE 1—Origin of Vibrio Fetus and Related Vibrio Strains

Group	Strain	Origin
I	UF	Bovine aborted fetus, Florida.
	426	Bovine aborted fetus, Florida.
	F	Bovine cervical mucus (with previous abortion), Florida.
	Ill.-1	Ovine aborted fetus, Illinois.
	Ill.-2	Ovine aborted fetus, Illinois.
	2221	Ovine aborted fetus, Montana.
II	AL	Bovine aborted fetus, Alabama.
	131	Bovine aborted fetus, Florida.
III	6722	Ovine aborted fetus, Montana.
	6725	Ovine aborted fetus, Montana.
IV	20	Bull semen, Maryland.
	4220	Bovine vaginal mucus, Maryland.
	10	Bovine vaginal mucus, Florida.
V	5494	Ram prepuce, Montana.
	5963	Ram prepuce, Montana.
	6411	Ram prepuce, Montana.
	6867	Ovine vaginal mucus, Montana.
VI	6961	Ovine vaginal mucus, Montana.
	6986	Ovine vaginal mucus, Montana.

a standard loopful of inoculum. At the same time, a loopful of the inoculum was streaked on duplicate Brucella agar plates which were then incubated as previously described. Thereafter, the plates were examined for variant colonies under 45 x magnification by a dissecting microscope, with obliquely transmitted light. If variation had occurred, at least 100 colonies were counted and the percentage of variant colonies was determined. A minimum of 12 and a maximum of 21 weekly transfers were accomplished with individual strains, and the colonial type and cellular morphology were determined each time in the cultures from which the transfers were made.

Antigens were prepared by propagation of the cultures on Brucella agar in 16-ounce prescription bottles. Plain agar (5 Gm.) was added to each liter of medium to increase solidity. The bottles were incubated in the manner previously described. Formolized antigens were prepared by suspending the cells in 0.3 per cent formaldehyde solution containing 0.85 per cent sodium chloride. Heat-treated antigens were prepared by heating washed cells in a boiling water bath for two hours, washing twice, and resuspending the cells in 0.3 per cent formaldehyde-saline solution. The concentrated antigens were stored in the refrigerator. Prior to use, the antigens were standardized to a density of approximately 45 per cent light transmission in formaldehyde-saline solution, using a Leitz photoelectric colorimeter with a 550 mμ green filter. This density approximated tube three of the McFarland nephelometer. Antiserums were prepared by inoculating rabbits intravenously, four times during a 21-day period, with approximately 10^7 Vibrio cells. Serum samples were obtained from cow AL on the day of abortion and at varied intervals for approximately six months thereafter. The blood samples were held at room temperature for several hours, after

TABLE 2—Colonial and Cellular Dissociation of Freshly Isolated Vibrio Fetus and Related Vibrio Strains

SOURCE OF STRAINS	GROUP	NUMBER AND ORIGIN OF STRAINS	COLONIAL FORM — APPARENT CELLULAR MORPHOLOGY		NUMBER OF WEEKLY TRANSFER
			ISOLATED	ON SUBSEQUENT TRANSFERS	
ABORTED FETUSES	I	3 (BOVINE) 3 (OVINE)	SMOOTH	GRANULAR SMOOTH → CUT-GLASS → MUCOID → ROUGH	21
	II	2 (BOVINE)	CUT-GLASS	CUT-GLASS	18
	III	2 (OVINE)	SMOOTH	GRANULAR SMOOTH → ATYPICAL MUCOID	12
GENITAL TRACTS OF NORMAL ANIMALS	IV	3 (BOVINE)	MUCOID CUT-GLASS	MUCOID CUT-GLASS → OCCASIONALLY GRANULAR SMOOTH	15
	V	4 (OVINE)	SMOOTH	SMOOTH → MUCOID	12
	VI	2 (OVINE)	SMOOTH	GRANULAR SMOOTH → MUCOID → CUT-GLASS	12

* *Vibrio fetus* strain F was isolated from the cervical mucus of a cow which aborted.

which they were placed in the refrigerator overnight. The serum was collected the following day and preserved at —20 C. until tested.

The method described by Kuzdas and Morse[6] was used to determine the tolerance of cultures to 3.5 per cent sodium chloride. The agglutination test used was previously described.[25] Catalase activity was determined on washed suspensions of the respective variants, obtained after approximately 60 hours' growth on solid Brucella agar. The density of the suspension was adjusted to approximate tube two of the McFarland standard. The suspension (10 ml.) was placed in a 16- by 150-mm. culture tube equipped with a rubber stopper, from which a gas delivery tube extended for 20 cm. into an inverted culture tube of similar size, filled with water and situated in a water bath at 25 C. Then 10 ml. of 3.0 per cent H_2O_2 was added to the tube containing the bacterial suspension and the system was closed. After 30 minutes at room temperature, the amount of oxygen produced by catalase activity was recorded as the number of milliliters of water displaced in the inverted tube. The variants capable of liberating a minimum of 1 ml. of oxygen were considered catalase-positive. Hydrogen sulfide determinations were performed by propagation of selected variants on Brucella agar slants, provided with lead acetate saturated, dried, filter-paper strips. Duplicate inoculums were carried on Brucella agar plates to ascertain the colonial purity of the respective variants. The cultures were incubated as described above. A darkening of the lead acetate strips after 72 hours was considered evidence of hydrogen sulfide production.

RESULTS

The origins of 19 Vibrio strains used in this study are shown (table 1). On the basis of the origin of the culture, colonial form originally isolated or demonstrated in first subculture, deviation on successive generations, catalase and H_2S activity, and tolerance of 3.5 per cent sodium chloride, the strains were classified into six groups (tables 2, 3). The strains of the first three groups originated from aborted fetuses, with the exception of strain F, which was isolated from the cervical mucosa of a cow which aborted. All of these cultures were catalase-positive and H_2S-negative and did not grow in the presence of 3.5 per cent sodium chloride. The cultures of groups IV, V, and VI were isolated from the genital tracts of apparently normal animals. The strains of groups IV and V were catalase-negative, H_2S-positive, and propagated in the presence of 3.5 per cent sodium chloride. These characteristics would place them in the saprophytic classification. However, group VI consisted of strains that produced catalase, did not produce H_2S, and grew in the presence of 3.5 per cent NaCl. This would classify them as "true" *V. fetus* strains, on the basis of catalase activity and H_2S results, but not on the basis of growth in 3.5 per cent NaCl.

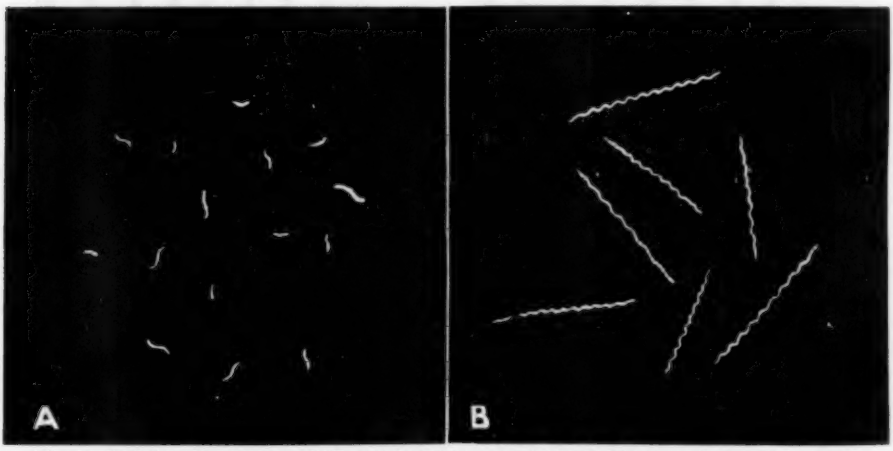


Fig. 1—Cellular Appearance of Two Colonial Types of Vibrio Fetus as Observed in Gram-Stained Impression Films from the Stomach Contents of Aborted Fetuses

A) Vibrio fetus, appearing as comma-shaped and incomplete S-shaped forms, approximately 1 to 1.5 μ in length. This organism was isolated in smooth colonial form of watery consistency.

B) Vibrio fetus, appearing as long, closely wound, spiral forms, approximately 3 to 7 μ in length. This organism was isolated in cut-glass colonial form of dewdrop appearance.

Group I.—This group was represented by six cultures, with equal numbers of bovine and ovine strains. These strains were initially isolated in smooth colonial form. The colonies were bluish gray, with an entire edge, and resembled dewdrops. The individual cells appeared as thin, curved, comma-shaped, and incomplete S-shaped forms, 1.0 to 1.5 μ in length, as illustrated (fig. 1A). On repeated subcultures, these smooth colonies assumed a slight surface granulation, *i.e.*, the typical dewdrop appearance was not maintained. The successive generations exhibited a progressive deviation from the parent type. The intensity of dissocation was dependent on the medium in which the organisms were propagated. The greatest number of nonsmooth variants were observed in tryptose semisolid broth and thioglycolate semisolid broth. Dissociation was less frequent on solid mediums. The general direction of dissociation to nonsmooth forms was: granular smooth to cut-glass to mucoid to rough. Description of nonsmooth colonial forms and corresponding cellular morphology was presented in a previous study.[11] The representative dissociation pattern of strains of this group is shown (fig. 2A, 2B).

Group II.—Two strains of this group were isolated in cut-glass colonial form from the stomachs of aborted bovine fetuses. The colonies were brownish gray-green, with occasional irregular edges, and with the appearance of a cut-glass surface having large facets. A watery consistency of these colonies was demonstrable by the tendency of the colony to flow away from the needle when touched. The organisms of this type were 2 to 5 μ long, with spiral morphology. The spirals

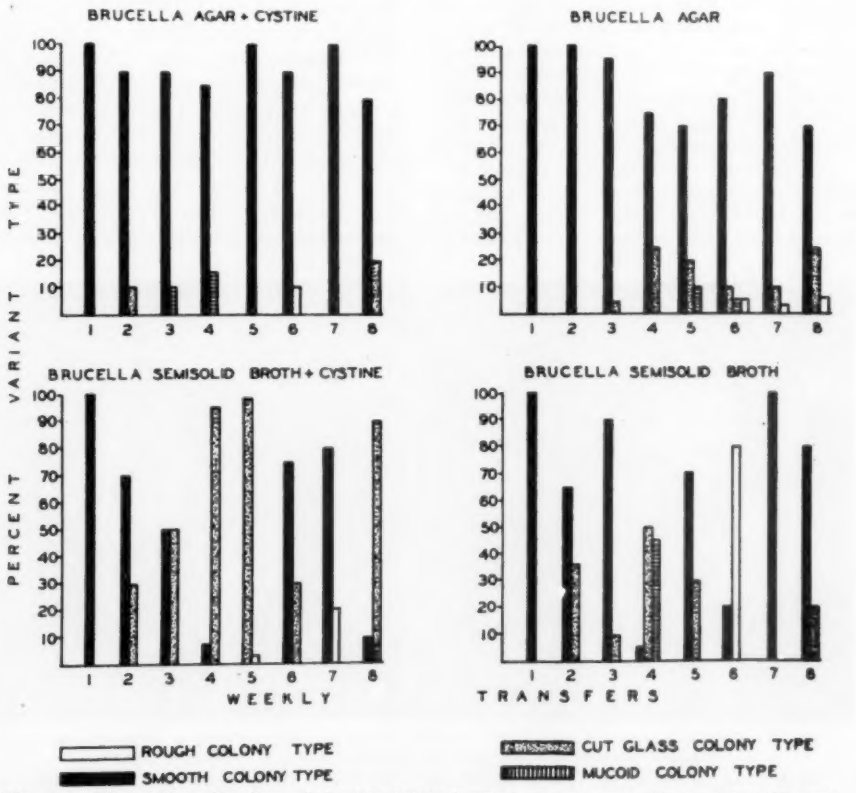


Fig. 2A—Dissociation direction of freshly isolated Vibrio fetus strain F on eight different culture mediums.

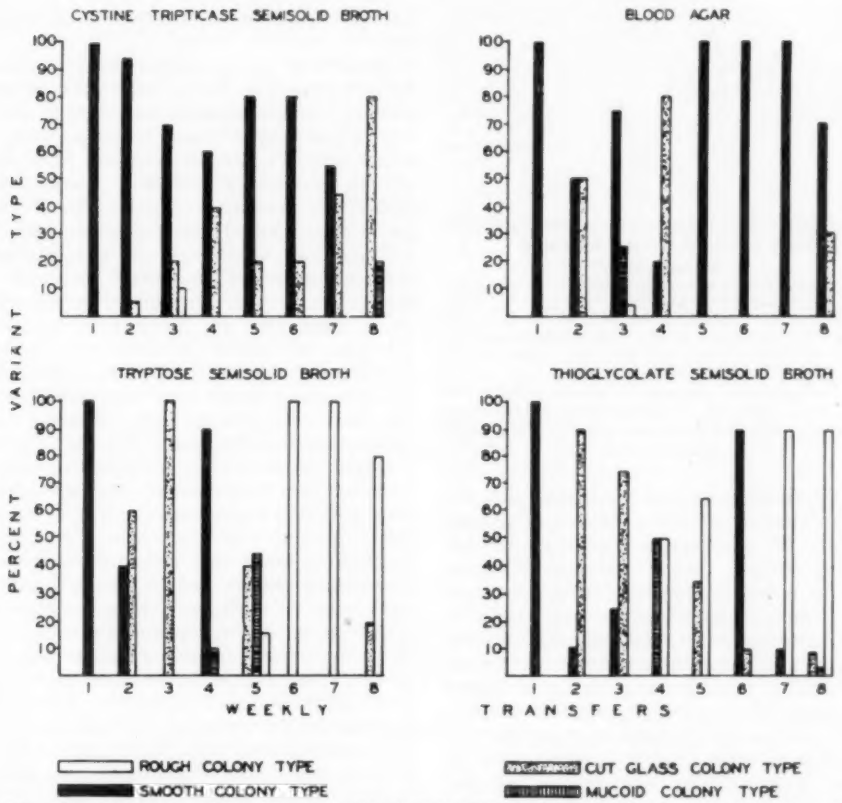
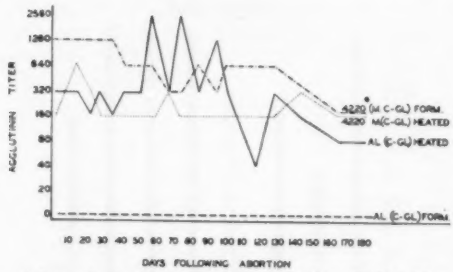


Fig. 2B—Dissociation direction of freshly isolated Vibrio fetus strain F on eight different culture mediums.

were closely wound with spaced internal granulations or septations, as illustrated (fig. 1B). Deviation from this parent colonial form was not observed on 18 subsequent transfers. The cellular morphology, however, changed to one consisting of long (2 to 6 μ), slightly wavy filaments.

Group III.—On isolation, the colonies of the two strains were smooth, entire-edged, fairly opaque, having a slight internal granulation. The cellular morphology appeared similar to previously described cells of smooth colonies. On successive generations, peculiar mucoid colonies developed. These were yellowish brown flat colonies containing a sharply defined, slightly raised, central daughter colony. The cellular morphology during the first 24 hours of growth appeared similar to smooth cell morphology. However, during the second and third days of incubation, the appearance of pleuropneumonia-like organisms of spherical and coccoid forms was observed. A reversion to smooth cell morphology was observed during early growth on subculture. Due to the resemblance of the cellular morphology to that of L-forms, originally described by Klieneberger[7] in cultures of *Streptobacillus moniliformis*, the term "L-form" was used in this study.

Group IV.—Cultures of this group were isolated as large, round, occasionally irregular edged, raised colonies with entire cut-glass surfaces, or yellowish centers and cut-glass peripheries. The colonies were distinguished by a mucoid consistency, demonstrated by a tendency to adhere to the medium when pulled with a loop. The organisms appeared as wavy filaments, 2 to 6 μ long, with no indication of true spiral forms. In subsequent cultures, the organ-



* Saprophytic Vibrio = catalase-negative, H_2S-positive, and has no growth in the presence of 0.85 per cent NaCl. M.C-GL = mucoid cut-glass variant; C-GL = cut-glass variant; Heated = heated for two hours in boiling water; Form. = formolized with 0.3 per cent formaldehyde-saline solution.

Fig. 3—Agglutinin response of serum, from a cow (AL) which aborted, to homologous Vibrio fetus strain AL and heterologous saprophytic Vibrio strain 4220.

isms generally retained the parent colonial and cellular appearance. However, smooth colonies of granular consistency, not exceeding 5.0 per cent of the total numbers, were observed during the last three transfers. Morphologically, the organisms of these colonies were indistinguishable from *V. fetus* of similar colonial types. Attempts to stabilize smooth variants by repeated selections failed. Mucoid cut-glass colonial types predominated upon each subculture.

Group V.—On initial isolation, the colonial and cellular appearance of the strains of this group were similar to cultures of group III. However, on subsequent transfers, these cultures dissociated to typical mucoid forms, with correlated cellular morphology. This type was described in a previous report.[11]

Group VI.—The colonial and cellular appearance of the original isolations and the dissociation patterns on successive generations of two strains of this group were similar to cultures of group I. Rough colonial forms were, however, not observed during the period of 12 subsequent transfers.

Results of serum-agglutination tests of serum samples from cow AL (which aborted) tested against homologous strain AL in cut-glass colonial form, saprophytic strain 4220 in mucoid cut-glass form, and smooth and rough antigens of strains UF and F, are presented (fig. 3, 4, 5). Formalin and heat-killed antigens were used. In no instance was there an agglutination reaction observed in serum dilutions as low as 1:10 with formolized homologous AL antigen (fig. 3). Agglutinins in serum dilutions of 1:40 to 1:2,560 were demonstrated when heat-treated homologous antigen was used. Sharp titer fluctuations were observed with this antigen. Similar reactions with less frequent titer fluctuations resulted when serums were tested against two antigen preparations of saprophytic strain 4220. Heat-treated antigen of the latter strain, however, was agglutinated in slightly lower serum dilutions. Smooth and rough antigens of strains UF and F, with some exceptions, detected serum agglutinins at higher serum dilutions with their formolized antigen preparations (fig. 4, 5). Heat-treated antigens of strain UF were slightly inferior to the formolized homologous antigens in their ability to detect serum agglutinins. Heat-treated rough antigen of strain F revealed no demonstrable serum agglutinins in any of the samples tested.

Data of cross-agglutination reactions between antigens of these four strains and their specific rabbit antiserums are shown (tables 4, 5). Formolized antigen of AL strain failed to agglutinate with homologous as well as heterologous antiserums (table 4). This antigen agglutinated, however, when used in heated form (table 5). On the basis of its formolized antigen, saprophytic strain 4220 revealed

TABLE 3—Physiological Characteristics of Freshly Isolated Vibrio Fetus and Related Vibrio Strains

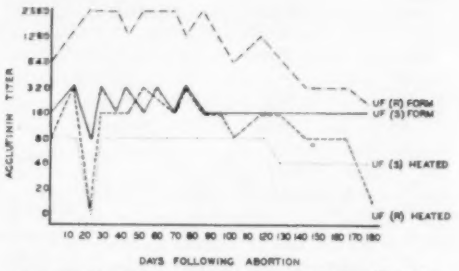
Source of strains	Group	No. and origin of strains	Catalase production	H_2S production	Growth in semi-solid broth containing 3.5% NaCl
Aborted fetuses	I, II, and III	5 (Bovine) 5 (Ovine)	+	−	−
Genital tracts of normal animals	IV and V	3 (Bovine) 4 (Ovine)	−	+	+
	VI	2 (Ovine)	+	−	+

negligible cross-reaction with rough variants of the heterologous strains. Heated antigen of this strain had a considerable degree of cross-reaction with antiserums prepared against formolized and heated cells of the three *V. fetus* strains. Strains F and UF were apparently serologically heterologous, with the exception of cross-reactions demonstrated with rough, heated antigens. Strain F exhibited a cross-reaction with antiserum of strain AL, mainly on the basis of its smooth, formolized and heated antigens.

DISCUSSION

The data presented indicate that: (1) there are at least two *in vivo* existing *V. fetus* colonial types, consisting of smooth and cut-glass forms; (2) the two types are characterized by concomitant cellular morphology and basic differences in ability to induce the formation of antibodies in rabbits; (3) within the bovine species, saprophytic Vibrio strains were distinguished from *V. fetus* strains by possessing relatively stable mucoid colonial form, a difference which was not demonstrated by the strains of ovine origin; and (4) successive generations of two colonial types exhibited a progressive colonial deviation from the parent type in the case of the smooth form, while the cut-glass type remained relatively stable.

While the cellular morphology of the smooth colonial form corresponded closely with the classical description of *V. fetus*, the cell form of the cut-glass type resembled the morphology which, by the majority of workers, is considered as nonsmooth and undesirable for antigen preparation. The possibility that the cut-glass colonial form represented a mutant of the first generation *in vitro* is lessened by the fact that impression smears made from the stomach contents of an aborted fetus (from which the cut-glass colonial form was isolated), revealed long, spiral cell forms, which were characteristic of freshly isolated cut-glass colonies.



S = smooth variant; R = rough variant; Heated = heated for two hours in boiling water; Form. = formolized with 0.3 per cent formaldehyde-saline solution.

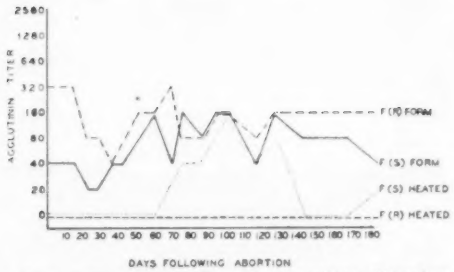
Fig. 4—Agglutinin response of serum, from a cow (AL) which aborted, to the variants of heterologous Vibrio fetus strain UF antigens.

A more important finding is the failure of formolized antigen of strain AL, prepared from cut-glass colonial type of freshly isolated culture, to detect the homologous antibody present in the serum of a cow which aborted. Several workers have failed to demonstrate agglutinating antibody in the serum of cattle from which *V. fetus* was cultured from the genital tracts.[6, 9, 10, 14] Ristic *et al.*[12] called attention to lack of demonstrable agglutinins in the serum of a cow which aborted when the serum was tested against formalin-killed *V. fetus* antigen, prepared from the cells isolated from an aborted fetus of this

TABLE 4—Cross-Agglutination Reactions Among Formolized Variant Antigens of Vibrio Fetus and Related Vibrio Strains and Their Antiserums

Formolized antigen			Antiserums *						Normal serums	
					UF		F			
Strain	Vibrio type	Variant	AL, cut-glass	4220, mucoid cut-glass	Smooth	Rough	Smooth	Rough	Rabbit	Heifer
AL	*V. fetus*	Cut-glass	0 **	0	0	0	0	0	0	0
4220	Saprophytic Vibrio †	Mucoid cut-glass	0	5,120	0	40	0	80	0	0
UF	*V. fetus*	Smooth	0	0	41,000	2,560	40	0	0	0
		Rough	0	0	1,280	82,000	0	40	0	80
F	*V. fetus*	Smooth	640	0	0	0	10,240	320	0	0
		Rough	0	0	80	0	160	10,240	0	40

* Prepared in rabbits against formolized cells; † saprophytic = catalase-negative, H_2S-positive, and capable of growth in the presence of 3.5 per cent NaCl; ** titer expressed as reciprocal of serum dilution.



S = smooth variant; R = rough variant; Heated = heated for two hours in boiling water; Form. = formolized with 0.3 per cent formaldehyde-saline solution.

Fig. 5—Agglutinin response of serum, from a cow (AL) which aborted, to heterologous variants of Vibrio fetus strain F antigens.

animal. Repeated agglutination tests, conducted at varied intervals over a period of 86 days following abortion, failed to demonstrate agglutinating antibody. The fact that heat-treated antigen strain AL, of cut-glass colonial form, was capable of detecting a considerable amount of antibody in the serum of the animal which aborted (from which aborted fetus the strain was isolated) indicates the presence of an antigenically less active, heat-labile portion on the cell of the cut-glass form. It is possible that the lack of demonstrable agglutinins in the serum of some of the described *V. fetus*-infected cattle might have resulted from the use of formolized antigens prepared from cells of the cut-glass colonial form. The serological inactivity and immunological inefficacy of the heat-labile component on the cells arising from cut-glass forms is further substantiated by agglutination test results, using specific antiserums produced in rabbits against two homologous antigens. Agglutinins were demonstrated with heat-treated antigens, while there was no reaction when formolized cells were used as agglutinating antigen.

A further point of interest is the serological cross-reaction of heat-treated antigen of saprophytic Vibrio strain 4220, which is catalase-negative, H_2S-positive, and propagates in the presence of 3.5 per cent sodium chloride, and strain AL (isolated from an aborted fetus), which is catalase-positive, H_2S-negative, and does not grow in the presence of 3.5 per cent sodium chloride. The common characteristic of the two strains is that both were initially isolated in a cut-glass colonial form and, with slight exceptions, revealed no colonial deviation from this form on successive generations in culture mediums. However, the cut-glass colonies of strain 4220 were of mucoid character, as distinguished from strain AL cut-glass colonies, which were of watery consistency. The mucoid cell surface component of strain 4220 and serologically inactive heat-labile cell portion of strain AL might have been responsible for the serological differences between the two strains, with reference to the formolized antigens and their specific rabbit antiserums.

From the present and previous studies, it appears, on the basis of their colonial dissociation patterns, that the majority of *V. fetus* strains belong to group I. The infrequency with which *V. fetus* is observed in a stable cut-glass form may arise from the possibility that the organisms of this colonial type tend to dissociate *in vivo* to the cut-glass type of mucoid character.

TABLE 5—Cross-Agglutination Reactions Among Heat-Treated Variant Antigens of Vibrio Fetus and Related Vibrio Strains and Their Antiserums

Heated antigen			Antiserums *						Normal serums	
					UF		F			
Strain	Vibrio type	Variant	AL, cut-glass	4220, mucoid cut-glass	Smooth	Rough	Smooth	Rough	Rabbit	Heifer
AL	*V. fetus*	Cut-glass	1280 **	320	2,560	1,280	2,560	1,280	40	40
4220	Saprophytic † Vibrio	Mucoid cut-glass	160	1,280	160	160	40	80	0	0
UF	*V. fetus*	Smooth	0	160	5,120	2,560	0	2,560	0	0
		Rough	0	320	640	5,120	0	2,560	0	0
F	*V. fetus*	Smooth	160	0	0	0	10,240	1,280	20	0
		Rough	40	0	0	1,280	2,560	1,280	0	0

* Prepared in rabbits against heated cells; † saprophytic = catalase-negative, H_2S-positive, and capable of growth in the presence of 3.5 per cent NaCl; ** titer expressed as reciprocal of serum dilution.

Bond [1] reported that mucoid variants of *V. fetus* behave, biochemically, similarly to saprophytic Vibrio. In our experience, prolonged cultivation of either smooth or nonsmooth variants on solid mediums resulted in a loss of their ability to produce catalase. However, on a single transfer of such cells to a semisolid medium the ability to produce catalase was regained. No change was observed in regard to H_2S production.

The L-type cells observed in successive transfers of two strains in group III resembled the morphology lately reported in several bacterial species, including *Vibrio comma*. The organisms arising from the L-type colonies have been claimed to represent special phases in a reproductive cycle of bacteria.[3,4] Groups IV and V, which represented strains presently designated as "saprophytic," were characterized by retaining, or assuming on successive transfers, the mucoid consistency. Two strains of group VI shared, to a certain extent, the colonial and the biochemical characteristics with *V. fetus* and saprophytic Vibrio strains. Of interest is the fact that these strains originated from animals with no history of reproductive disorder.

The dissociation pattern in successive generations of strain F, as demonstrated (fig. 2A, 2B), was typical for the strains of group I. Apparently none of the eight mediums used were capable of stabilizing the smooth colonial form. Dissociation, however, was more frequent in semisolid mediums, which supported better growth, than on solid mediums. From the practical point of view, it appears that the smooth type needed for antigen preparation is maintained more easily on solid mediums by means of periodic selection of colonies.

SUMMARY

On the basis of the colonial and cellular characteristics of the parent culture and dissociation pattern in successive generations, 19 *Vibrio fetus* and other Vibrio strains of bovine and ovine origin were classified into six groups. The evidence presented indicates the *in vivo* existence of two *V. fetus* colonial types with concomitant cellular and antigenic characteristics. Hydrogen sulfide-positive and catalase-negative saprophytic Vibrio strains of bovine origin were distinguished from *V. fetus* strains on the basis of their colonial characteristics. Similar differences in colonial characteristics were not demonstrated in saprophytic strains of ovine origin. A saprophytic Vibrio strain of bovine origin shared heat-stable antigens with *V. fetus* strains originally isolated in cut-glass and smooth colonial form, on the basis of specific rabbit antiserums.

Serum agglutinins of a cow which aborted were detected, during the six-month period after abortion, with formolized and heated antigens of a saprophytic Vibrio strain and heated but nonformolized antigen of homologous *V. fetus* strain.

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A Comparison of the Effect of Catalase-Positive and Catalase-Negative Vibrio on the Fertility of Cattle

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TWO TYPES of Vibrio, which are commonly isolated from the reproductive tracts of cattle, have been described in a preliminary report.[2] One was found to be catalase-positive (C+ Vibrio), and the other catalase-negative (C— Vibrio). Reports have shown that C+ Vibrio is pathogenic and definitely interferes with conception in cattle;[1,5-10] however, the significance of C— Vibrio has not been definitely established.[2,4]

The purpose of this paper is to compare the transmissibility, persistence of infection, and effect on conception of C— and C+ Vibrio by natural service of susceptible cows to infected bulls. Some of this information has been presented in summary form.[6]

MATERIALS AND METHODS

Vibriosis was studied in four groups of cattle.

The first group consisted of 65 virgin heifers and 72 cows that had been bred. Virgin heifers, 18 to 20 months old, were continuously added to the breeding herd as replacements. Each female was given a 60-day sexual rest period following parturition. The breeding performance of the herd, which included a total of 153 pregnancies, was studied over a period of nearly two years. Six bulls used for breeding were infected with C— Vibrio and each was assigned specific females for each calving cycle.

The Vibrio status of females was determined, before and after breeding, by bacteriological examination of vaginal mucus,[3] which was collected from heifers near the time of sexual maturity and a short time before breeding, and from all cows about 30 days after parturition. Postbreeding collections were made from all females within 30 days after service and at the time of examination for pregnancy. Additional mucous samples were collected at intervals of one to two months from repeat breeders, from those that aborted, and from those that became infected.

The second group consisted of 27 virgin heifers and 91 cows that had calved in 1954. These animals were assigned for breeding during April, May, and June of 1955, to 9 C— Vibrio-infected bulls and 2 Vibrio-free bulls. Thirteen virgin heifers and 14 cows were bred to the Vibrio-free bulls, and 14 virgin heifers and 77 cows were bred to the C— Vibrio-infected bulls. The Vibrio status of these females was determined by bacteriological examination of vaginal mucus, which was collected monthly from April through August.

The third group consisted of 6 virgin heifers and 3 bulls that had not been used for service. Each animal was placed in individual isolation. The Vibrio status of the animals was determined by bacteriological examination of vaginal mucus, collected weekly, and of preputial and semen samples, collected at irregular intervals. After six weeks of examinations, the animals were assigned for breeding.

The 6 virgin heifers in the fourth group were bred to a bull which was naturally infected with C— Vibrio and experimentally infected with C+ Vibrio. This bull was infected, by preputial injection, with the second cultural transfer of C+ Vibrio, which was originally isolated from an aborted fetus. Heifers were bred to this bull until C+ Vibrio infection was established, at which time they were assigned to bulls that were free of C+ Vibrio.

A comparison was made of the antigenic characteristics of the various strains of C— Vibrio isolated from the four groups of cattle. Each of two or more strains, isolated from each group of cattle, was inoculated into a rabbit for hyperimmunization. The antiserum from each rabbit was tested for agglutinability with homologous and heterologous antigens.

RESULTS

In group 1, Vibrio could not be isolated from vaginal mucus obtained from 65 heifers prior to breeding. The rate of transmission of C— Vibrio and its effect on conception in susceptible females bred to infected bulls are presented (table 1). The incidence of C— Vibrio infection was low and, in most cases, was of short duration. Catalase-negative Vibrio was isolated once from each of 10 cows, twice in six months from 1 cow, three times in one year from each of 2 cows, and five times over a period of two years from 1 cow. Of the 15 C— Vibrio-infected cows, 6 required

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TABLE 1—Spread of Catalase-Negative Vibrio Within a Herd, Using Natural Service to Infected Bulls (Group 1)

	Bull (No.)	No. of females	No. of females infected	No. of pregnancies	Services per conception
	1	9	1	9	1.6
	2	25	1	21	1.8
	3	30	5	38	1.4
	4	33	1	44	1.4
	5	20	4	20	1.8
	6	20	3	21	1.8
Totals	6	137	15	153	1.6

one service each per conception, 6 required an average of three services each, and 3 failed to conceive. The classification of these 3 as failures may be misleading, because they were removed for reasons other than sterility.

In group 2, the number of services per conception of susceptible females bred to C— Vibrio-infected bulls was compared with those of females bred to Vibrio-free bulls (table 2). There was no significant difference in the average number of services required for each conception. Catalase-negative Vibrio was isolated from vaginal mucus of 11 of 91 females bred to 9 infected bulls. However, 3 of the 9 infected bulls did not transmit C— Vibrio to 27 females, whereas the remaining 6 transmitted it to only 11 of 64 females. The causative organism was isolated from 4, once each; from 4, twice each; and from 3, three times each. Moreover, the average number of services per conception was no higher in the 11 infected animals than in the 91 noninfected animals. No Vibrio was isolated from any of the females bred to Vibrio-free bulls, except from 1 heifer. Catalase-negative Vibrio was isolated from this heifer after her third service; unfortunately, there is no logical explanation for the presence of the organism.

In group 3, prebreeding examinations showed that the 3 bulls were infected with C— Vibrio and none of the 6 virgin heifers were infected with either C— or C+ Vibrio. Catalase-negative Vibrio was transmitted from the naturally infected bulls to 5 of the heifers (table 3). These heifers required an average of 3.4 services per conception, whereas the noninfected heifer failed to conceive after 10.0 services. Only 2 of the 3 bulls would serve the heifers. One of these 2 bulls had poor quality semen and was bred to 3 heifers, whereas the other bull had good quality semen and was bred to 5 heifers, 2 of which had been bred to the previous bull. The rate of infection and the number of services per conception were considerably higher in the heifers in this group than in the 14 heifers in group 2 (table 2). However, only one or two isolations were made from each female except heifer 3, from which Vibrio was isolated seven times between 60 and 156 days after her first service.

TABLE 2—Comparison of Breeding Performances of Heifers and Cows Bred to Catalase-Negative, Vibrio-Infected and Vibrio-Free Bulls (Group 2)

	Heifers				Cows			
Breeding bulls	No. bred	No. infected	No. pregnant	Services per conception	No. bred	No. infected	No. pregnant	Services per conception
C— Vibrio-infected	14	1	13	1.7	77	10	73	1.6
Vibrio-free	13	1	12	1.9	14	0	14	1.1

In group 4, the transmissibility and course of each type of Vibrio infection in the 6 susceptible heifers, bred to a bull naturally infected with C— Vibrio and experimentally infected with C+ Vibrio, are presented (table 4). Only 4 of the 6 heifers became infected with C— Vibrio,

TABLE 3—Spread of Catalase-Negative Vibrio from Naturally Infected Bulls to Virgin Heifers (Group 3)

Heifer (No.)	No. of services	Days required for conception	No. of isolations		Incubation period (days)	Estrous cycle (av. in days)
			Before pregnancy	After pregnancy		
1	1	1	0	1	33	....
2	10	0	0	..	....	25
3	5	107	3	4	60	27
4	3	40	0	2	65	20
5	6	129	0	1	180	26
6	2	22	1	0	9	22

TABLE 4—Transmission of Catalase-Positive and Catalase-Negative Vibrio from an Infected Bull to Susceptible Females by Natural Service (Group 4)

Heifer (No.)	No. of services	Days required for conception	C+ Vibrio isolations after exposure at first service			C— Vibrio isolations after exposure at first service			Estrous cycle (av. in days)
			(No.)	First isolation (days)	Last isolation (days)	(No.)	First isolation (days)	Last isolation (days)	
7	2	291	26	4	219	0	.....	.....	32
8	10	284	27	11	320	3	78	321	31
9	13	374	34	12	464	5	270	393	31
10	4	87	19	14	245	0	.....	.....	29
11	7	203	24	7	400	1	379	379	37
12	2	55	7	3	194	2	120	120	55

whereas all of them became infected with C+ Vibrio. In addition, infection caused by C+ Vibrio spread more rapidly and persisted longer than that produced by C— Vibrio. All of the heifers became infected and none conceived following the first service to the C+ Vibrio-infected bulls.

Heifer 7 was not rebred until ten months after her first service. During this time she had nine estrous cycles. She became free of C+ Vibrio after ten months. Following this rest period, she conceived from one service to a bull free of both C— and C+ Vibrio. Four heifers (No. 8, 9, 10, 12) were bred at each estrus to the original bull, as well as to bulls free of C+ Vibrio but naturally infected with C— Vibrio, and each required an average of six additional services per conception, covering a period of approximately 200 days. Heifers 8 and 9 were pregnant 30 days after their last service, and apparently had early fetal resorptions. Heifer 8 became free of C+ Vibrio 40 days following conception, but heifer 9 remained infected. Heifer 10 remained infected 161 days after conception, but calved normally. Heifer 12 conceived, but continued to be infected with C+ Vibrio. Heifer 11 was likewise bred to the bulls used on the other 4 heifers and conceived at the time she became negative for C+ Vibrio, which was 203 days after her first service. She remained free of both types of infection for 150 days, at which time she was accidentally bred by the experimentally infected bull while being used as a dummy for semen collection. Catalase-positive Vibrio was isolated on the seventh day, and C— Vibrio was isolated on the twenty-eighth day after the accidental service. She remained infected with C+ Vibrio until 28 days after giving birth to a normal calf.

A total of 211 vaginal mucous samples collected from the 6 heifers were cultured, 158 (75%) of which were suitable for isolation and identification of Vibrio. Catalase-positive Vibrio was isolated from 85 per cent of the 158 samples, C— Vibrio from 5 per cent, and both types from 2 per cent. Vibrio was not obtained from the remaining 8 per cent.

Semen collected from the bulls used to breed these heifers was of satisfactory quality.

One of the C+ Vibrio-free bulls used for breeding the C+ Vibrio-infected heifers became infected after six services, and the other remained free of C+ Vibrio after 13 services. Semen from the experimentally infected bull remained positive for both types of Vibrio for 18 months.

Two distinct serological types of C— Vibrio were isolated from cattle in group 1. Only one of these types was isolated from cattle in groups 2, 3, and 4.

DISCUSSION

It was thought that the bull was the disseminator of vibriosis of cattle. In our work on infected cattle herds with impaired fertility, two types of Vibrio have been isolated from the reproductive tracts of both males and females being used for breeding. Impaired fertility was frequently associated with C+ Vibrio, but this was not necessarily true with C— Vibrio. Consequently, the significance of this latter type of Vibrio remained questionable until the present work was completed.

With few exceptions, C— Vibrio was isolated from both preputial material and semen collected from bulls. Furthermore, it occurs frequently as a natural infection in the prepuces of bulls that have never been used for service.

Most bulls infected with C— Vibrio had satisfactory breeding records, in contrast to those infected with C+ Vibrio. No

other clinical or pathological manifestations were associated with either type of infection. The bull which was artificially infected with C+ Vibrio recovered from infection, spontaneously, after 18 months; however, he remained infected with C— Vibrio. None of the C— Vibrio-infected bulls, kept under close observation, have recovered spontaneously. On postmortem examination, both types of Vibrio have been isolated only from the prepuce.

Catalase-negative Vibrio, like the C+ type, has been isolated from vaginal mucus of females only after breeding. The incidence of cultural isolations of C— Vibrio from vaginal mucus was low, and the incubation period usually varied from ten to 30 days, but was ten months or longer in a few cases. A single isolation was made in approximately one half of the infected females, and two to six isolations were made in the other half. Multiple isolations were made during periods which varied from a few weeks to 12 months or more. On postmortem examination, C— Vibrio has seldom been isolated from the vaginas of females that had been bred to bulls infected with C— Vibrio.

Some C— Vibrio-infected bulls apparently fail to infect a single female during a breeding season, whereas others may infect 5 to 20 per cent of the females. The conception rate of infected females was similar to that of noninfected females bred to the same infected bull. Moreover, it was usually similar to that in Vibrio-free females bred to Vibrio-free bulls.

Catalase-positive Vibrio was spread more readily than C— Vibrio to susceptible females, and it had a shorter incubation period and a longer course of infection. Another outstanding difference was that cattle infected with C+ Vibrio required considerably more services per conception that those infected with C— Vibrio.

SUMMARY

The relationship of vibriosis and infertility of cattle was discussed.

Catalase-negative (C—) Vibrio was isolated frequently from bulls that had not been used for service, but it was never isolated from virgin heifers.

Catalase-negative Vibrio was seldom spread from naturally infected bulls to susceptible heifers and cows by natural service. Except in the 6 heifers in group 3, the conception rate was similar to that in Vibrio-free cattle. Furthermore, infection was of short duration in most females, while it was persistent in bulls.

Catalase-positive (C+) Vibrio was not isolated from virgin bulls and heifers. It was transmitted from an infected bull to susceptible heifers and from infected heifers to a susceptible bull. This type of Vibrio prevented conception, causing prolonged infertility, a condition frequently associated with *Vibrio fetus*.

Bulls can be infected with C+ and C— Vibrio simultaneously. No gross pathological changes or clinical manifestations were associated with vibriosis of bulls.

Two antigenically different C— Vibrios were isolated from naturally infected cattle.

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Several Blood Constituents of Five Breeds of Dairy Cattle in Guatemala

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IN ORDER to increase the production of milk in countries of the American tropics, governments, agricultural organizations, and private breeders are importing increased numbers of purebred dairy cattle from the United States and Canada. Some of these cattle are maintained as purebred herds; others are crossed with selected native cattle.

Many of these imported animals die and, often, they produce less milk than the native cattle. Although the reasons for this high mortality and inefficient production are numerous and complicated, there is no doubt that the existing low plane of nutrition is an important factor. Rusoff and Piercy [13] have stressed the value of blood data as an aid in determining the nutritional status of dairy animals. This report, a study of 12 essential blood constituents of dairy cattle considered representative of better managed herds in Guatemala, concludes the series initiated by Squibb *et al.*[15] which were designed to provide reference data on farm animals of this tropical area.

TABLE 1—Levels of Several Blood Constituents of Five Breeds of Dairy Calves and Cows in Guatemala

Constituent		Native		Jersey		Brown Swiss		Holstein-Friesian		Ayrshire	
		Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
SERUM											
No. of animals	Calves	40		5		47		17		22	
	Cows	15		18		49		30		10	
Total proteins	Calves	6.21	0.62	6.33	0.21	6.19	0.28	6.75	0.11	6.50	2.00
(Gm./100 ml.)	Cows	.....	.....	7.27	0.18	7.23	0.13	.....	.....	.....	.....
Riboflavin	Calves	1.44	0.59	2.01	0.24	1.97	0.61	0.86	0.08	1.10	0.24
(μg./100 ml.)	Cows	1.39	1.03	1.67	0.57	3.00	1.02	2.56	0.74	1.59	0.97
Ascorbic acid	Calves	0.60	0.23	1.44	0.21	0.81	0.14	1.10	0.05	0.50	0.09
(μg./100 ml.)	Cows	.....	.....	1.20	0.08	1.06	0.11	.....	.....	.....	.....
Carotene	Calves	77.00	43.00	478.00	51.00	304.00	95.00	79.00	8.00	293.00	184.00
(μg./100 ml.)	Cows	900.00	225.00	1,097.00	101.00	666.00	152.00	886.00	31.00	893.00	214.00
Vitamin A	Calves	12.00	5.50	24.00	2.60	23.00	28.70	12.00	0.90	16.00	13.90
(μg./100 ml.)	Cows	51.00	26.60	58.00	9.70	34.00	12.80	47.00	7.00	40.00	14.40
Total tocopherols	Calves	0.45	0.16	0.27	0.02	0.50	0.12	0.16	0.03	0.33	0.18
(μg./100 ml.)	Cows	.....	.....	0.60	0.34	0.91	0.04	.....	.....	.....	.....
Alkaline phosphatase	Calves	9.76	3.14	5.70	0.85	5.48	0.91	2.03	0.14	3.77	2.91
(mM./1/hr.)	Cows	2.30	0.46	1.54	0.58	2.31	1.00	2.62	2.10	4.62	3.21
Calcium	Calves	15.53	2.23	.....	.....	10.13	0.81	.....	.....	9.12	0.74
(mg./100 ml.)	Cows	.....	.....	6.89	0.75	10.61	1.58	10.15	1.21	9.32	1.14
Phosphorus	Calves	9.29	1.07	.....	.....	7.03	0.58	.....	.....	8.31	1.16
(mg./100 ml.)	Cows	.....	.....	4.15	0.34	6.17	1.04	6.90	0.85	6.03	0.86
WHOLE BLOOD											
No. of animals	Calves	27		.....	.....	10		17		.....	.....
Hemoglobin (Gm./100 ml.)	Calves	12.9	1.8	.....	.....	10.1	0.6	11.8	0.4	.....	.....
Erythrocytes (millions/mm.^{3}) count	Calves	11.5	2.4	.....	.....	8.4	0.5	9.7	0.5	.....	.....
Hematocrit (%)	Calves	53.0	7.0	.....	.....	34.0	1.4	31.0	0.2	.....	.....

MATERIALS AND METHODS

From April, 1952, to May, 1954, blood samples were obtained from several herds of Jerseys, Holstein-Friesians, and Ayrshires of highland areas (elevation over 3,000 ft.) and Brown Swiss and native breeds of lowland areas (from sea level to 1,500 ft.). All cattle had access to either green soilage or pasture. The cows were 3 to 5 years of age and were in the third to fifth month of lactation. The calves ranged from 2 to 5 months of age and were all nursing. There was no rela-

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tionship between the cows and the calves used in this study.

Approximately 15 ml. of blood was drawn from the jugular vein and permitted to coagulate to yield the serum. An additional 2 ml. was collected in tubes containing an anticoagulant. All samples were immediately placed in a small ice box and shipped by air to the laboratory. Within five hours after blood samples were collected, analyses were begun for protein,[10] riboflavin,[4] ascorbic acid,[11] carotenoids, and vitamin A,[1] tocopherols,[12] alkaline phosphatase,[1] calcium,[8] and phosphorus.[8]

RESULTS AND DISCUSSION

Since only better managed herds were sampled, it was anticipated that the blood levels for the various constituents studied would be within normal limits. The serum protein values of the cows (table 1) would be even closer to those given by Dukes[5] for plasma protein if the fibrinogen content of the plasma were added. The vitamin A and carotene blood levels were adequate and similar to those reported elsewhere.[6, 14, 17] Obviously, these cattle, fed ample quantities of fresh green soilage or given access to green pastures, had an adequate intake of carotene. The relatively lower serum carotene and vitamin A levels of the native and Holstein-Friesian calves suggest that they were consuming poorer quality forage prior to the time the blood samples were drawn.

The ascorbic acid serum levels were, on the average, higher than those reported by Braun[3] for dairy cows on pasture. The serum alkaline phophatase and tocopherol values were similar to those previously reported[9, 15] as were the levels of calcium and phosphorus.[3, 13] The rumen and intestinal synthesis of riboflavin, which takes place in cattle, and dietary intake determine the serum levels (table 1) of this vitamin.

While the hemoglobin and hematocrit values and the erythrocyte counts of the purebred calves were similar to published values,[13, 18] the native calves had comparatively higher values for these blood constituents.

SUMMARY

Chemical analyses were made of several blood constituents of dairy cows and calves of five breeds which were considered representative of better managed herds in Guatemala. The data indicate that blood serum levels of total proteins, ascorbic acid, tocopherols, hemoglobin, erythrocytes, hematocrit, alkaline phosphatase, carotenoids, and vitamin A were similar to values published elsewhere as normal. Native calves raised in the tropic lowlands were observed to have higher hemoglobin and hematocrit values and erythrocyte counts.

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Esophageal Innervation and the Eructation Reflex in Sheep

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ERUCTATION is extremely important in the ruminant. Normal productivity, well being, and life itself are dependent upon its efficient functioning. When the eructation mechanism fails, bloat or tympanites develops. The economic loss caused by this condition is one of the most serious problems facing the livestock industry. Cattle are universally plagued with bloat, but sheep are affected only in certain areas.[28] Before the pathogenesis of bloat can be elucidated, the fundamental mechanisms of eructation must be more fully understood. Experiments were, therefore, designed to increase our knowledge of eructation.

REVIEW OF LITERATURE

Eructation and esophageal innervation have been studied in domestic and laboratory animals by several workers. Chauveau[2] stimulated the vagal roots, intracranially, in freshly slaughtered horses. This produced immediate contraction of the esophagus down to the area adjacent to the heart. There was delayed contraction of the lower esophagus and stomach. The contraction of the lower part was "vermicular," not tetanic like that in the upper part. The difference may be attributed to the fact that the lower part is smooth muscle and the upper part is striated. Stimulation of the cervical sympathetic nerve at the points where it anastomoses with the vagus had no effect on the esophagus.

When the vagus was stimulated at the thoracic inlet, only the portion of the esophagus caudal to the heart contracted, indicating that the recurrent nerve does not supply motor innervation to the cervical esophagus in the horse. On the other hand, stimulation of the esophageal division of the pharyngeal branch of the vagus caused contraction of the cervical esophagus. It also caused contraction of the terminal esophagus in a few cases.

Stimulation of the central end of one cervical vagus after cutting both vagi caused reflex contraction of the cervical esophagus. Other central stimulation experiments led Chauveau[2] to the conclusion that the branches of the recurrent nerve to the esophagus are sensory in the horse. Bilateral section of the esophageal division of the pharyngeal branch of the vagus paralyzed the cervical esophagus.

Experiments on the cow were limited, but Chauveau[2] concluded that the esophageal division of the pharyngeal branch of the vagus was motor and the recurrent nerve was sensory to the esophagus, as in the horse. Bilateral cervical vagotomy in the dog did not stop peristalsis in the cervical esophagus, although it paralyzed the thoracic esophagus. Chauveau thought that the dog differed from the horse in that the pharyngeal branch of the vagus was both afferent and efferent for the cervical esophagus. Thus, Chauveau demonstrated very early that the term "pharyngeal branch of the vagus" (ramus pharyngeus n. vagi), adopted from human anatomy, is not adequate for the domestic animals because the branch is not limited to the pharynx but also supplies an important part of the esophagus. A more suitable term, pharyngoesophageal nerve (PEN) was proposed much later by Hwang, Grossman, and Ivy.[17]

Langley[22] stated that the cardiac sphincter was controlled entirely by the vagus. He atropinized and curarized the rabbit to eliminate the stimulatory effects of the vagus and was able to demonstrate that the vagus also inhibited the cardiac sphincter.

Meltzer[26] repeated the experiments of Wild and of Mosso (cited by Meltzer[26]) in an effort to explain their contradictory conclusions with regard to esophageal peristalsis. Wild stopped the progress of peristalsis by compression, ligation, or division of the esophagus, and drew the conclusion that esophageal peristalsis is caused by peripheral reflexes. Mosso showed that the extrinsic nerves were necessary for propagation of a peristaltic wave and that they could transmit the wave to the distal stump of the transected esophagus. He concluded that the peristaltic reflexes of the esophagus are mediated by a center in the brain.

Meltzer's results[26] indicated that both peripheral and central reflexes were involved and that the apparent contradictions could be explained by the level of anesthesia employed. Wild obtained deep anesthesia with opium given intravenously and Mosso used light ether anesthesia. Meltzer stated, "with a moderate degree of anesthesia the center of deglutition is not materially constrained in its function, and the successive contractions of the different parts of the esophagus are attended

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to by the primary afferent impulse which started the entire act of deglutition."

Hwang, Grossman, and Ivy [17] have presented a comprehensive review of the literature and a sound anatomical and experimental study of esophageal innervation of the dog, cat, rabbit, monkey, guinea pig, and rat. Previous work by Hwang, Essex, and Mann [18] had shown that complete sympathetic ganglionectomy had no effect on the esophagus. Stimulation of the cranial cervical sympathetic ganglion produced no change in the esophagus. They confirmed Chauveau's observation [2] that bilateral cervical vagotomy did not stop peristalsis in the cervical esophagus in chronic experiments conducted on dogs and cats. This indicated that the recurrent nerve is not essential for motor activity of the cervical esophagus. Bilateral section of the pharyngoesophageal nerve (PEN) paralyzed the cervical esophagus. Central stimulation of one vagus with both vagi cut caused tetanic contraction of the cervical esophagus. This reflex was stopped by cutting the PEN on the same side as the stimulated vagus. They concluded that the PEN was the main, if not the only, innervation of the cervical esophagus in the dog and cat. In another part of their paper they stated that the cervical esophagus has a dual innervation from the PEN and the recurrent nerve. In the majority of dogs, stimulation of the PEN caused contraction of the whole cervical esophagus and stimulation of the recurrent nerve caused contraction of the caudal fourth or third. In about one fifth of the dogs, the area of overlap was higher up the neck: the PEN caused contraction of the cranial two thirds, and the recurrent nerve caused contraction of the caudal two thirds, or the entire cervical esophagus.

In cats, this reaction was more consistent than in dogs. Stimulation of the recurrent nerve caused contraction only of the thoracic esophagus and of about an inch of the lower cervical esophagus. In the *Macaca rhesus*, although stimulation of the external laryngeal nerve, a branch of the cranial laryngeal, caused contraction of the upper three fourths of the cervical esophagus, stimulation of the cervical vagus caused contraction of the whole esophagus. The innervation in the guinea pig and rat was like that in the monkey.

In the rabbit, stimulation of the cervical vagus caused contraction of the whole esophagus, but there was also a branch of the pharyngeal nerve hidden under the pharyngeal muscles. It caused contraction of the upper one half of the cervical esophagus reflexly, when the central end of the cut vagus was stimulated.

Stevens and Sellers [32] prepared cattle so that they could apply procaine through a tube to the dorsal vagal trunk in the thorax and stimulate above or below the block. They found that the procaine block did not affect the rate of eructation, but decreased the efficiency. Probably, afferent impulses for the reflex were carried in the ventral trunk. It has been shown that either abdominal trunk is capable of mediating enough eructation activity to prevent bloat (Duncan,[12] Habel [16]). The site of the block was too far caudad to interfere with the esophageal phase of eructation. The effect of the block was to reduce the transmission of stimuli from the stomach, and to stop some of the motor impulses to the stomach. This probably impaired the efficiency of the cardia-clearing contractions of the reticulum and rumen, and the eructation contraction of the rumen.

It is generally accepted that eructation is a reflex. This has been reported by Quin,[26] Cole *et al.*,[6] Mangold and Klein,[24] Hoflund,[15] and Weiss.[33] However, only a limited amount of work has been done to locate the reflex centers. Clark,[4] using 54 sheep, 7 goats, and 2 cows, studied the effects of vagotomy, decerebration, frontal lobectomy, and intracerebral injections of methanol on the motility of the rumen and reticulum, and on rumination. He concluded that a subcortical area of the brain, anterior to the infundibulum of the pituitary gland, was associated with the nervous control of rumination and motility of the rumen and reticulum, and that these functions were not controlled by the medulla. Bell and Lawn,[1] however, using electrical stimulation of the medulla, were able to produce responses of the rumen, reticulum, and esophagus. These responses were consistent and reproducible even after transection of the rhombencephalon at the pontine level and the spinal cord at the first cervical vertebra. Reticuloruminal responses were obtained by stimuli within the limits of 6 mm. rostral and 2 mm. caudal to the obex. The reactive loci were in the lateral region of the medulla, mainly in the dorsal part of the lateral reticular formation. Most of the esophageal responses were obtained from a similar anatomical location, but the reactive area lay between 3 and 7 mm. in front of the obex. The motor effect on the reticulorumen, elicited by central stimulation, is mediated via vagal nerves without any intramedullary decussation.

The receptors and the afferent limb of the eructation reflex are have received some attention. Wester, cited by Weiss,[33] observed that eructation can occur in an animal with an open fistula and concluded that an increased intraruminal pressure was not essential to initiate the reflex. Stålfors,[31] however, believed that gas pressure was an important part of the eructation-stimulating mechanism. Dougherty,[10] Nichols,[27] and Weiss [33] have shown that, when intraruminal pressure was increased by insufflation, an increase in ruminal activity and eructation was produced. Weiss [33] has shown that the number of eructation contractions, as compared with regular rumen contractions, increased during insufflation. He postulated that pressure receptors are located in the wall of the rumen, principally in the posterior dorsal blind sac, and that stimulation of these receptors initiates the eructation reflex. Iggo [19] has demonstrated impulses in afferent fibers of the cervical vagus of the anesthetized goat during distention of the reticular and ruminal sacs of the stomach. He has located some of the receptors by exploring the stomach with a probe and by pulling on strips of the stomach wall. In each case, he located them near the junction of the esophageal groove with the wall of the reticuloruminal sac. Digital com-

pression was an effective stimulus. He suggests that the receptors are tension receptors in series with the muscle. No evidence was found that visceral receptors are in parallel with muscle or that they act as so-called volume receptors. No attempt was made to associate these receptors with the eructation mechanism.

Mead, Cole, and Regan [25] and Cole, Mead, and Regan [7] advanced the theory that the presence of coarse material in the rumen stimulated the eructation reflex. They [7] reported some correlation between rumination and eructation and postulated that both acts were initiated by the same reflex. Clark [5] disagreed, arguing that it is difficult to conceive why coarse material, which would be expected to stimulate rumination, should also stimulate eructation. Schalk and Amadon [30] initiated the rumination reflex by scratching the mucosa of the reticulum. Dougherty and Meredith [12] have shown, through cinefluorographic methods, that an increase in gas pressure alone will greatly increase the rate of eructation.

There is some evidence [12] that the cardia functions less efficiently when covered by ingesta or fluid. However, in the cinefluorographic studies, when the cardia was covered or partially covered with fluid, eructation did occur, with a minimum of fluid entering the esophagus. The fluid was reswallowed before the next eructation. When the cardia was covered with fluid and the caudal end of the sheep was raised, the increased intraruminal pressure induced by insufflating a gas mixture through the rumen cannula distended the rumen and lowered the ingesta enough so that most of the fluid mass was kept away from the cardia. Dougherty and Habel [11] completely filled the rumen of a sheep with fluid and then applied a pressure of 40 mm. Hg by injecting water. Cinefluorographic studies were made during the time of increased pressure. The cardia and the functional sphincter in front of the diaphragm were first closed, but finally opened during the height of the pressure, permitting fluid to enter the esophagus. The cranial esophageal sphincter remained tightly closed and at no time did it permit fluid to enter the pharynx. It is possible that receptors located in the area of the cardia or in the esophagus may be involved in reflex inhibition of eructation.

DeWitt [9] described the myenteric and submucosal plexuses of the esophagus of the cat. The subepithelial plexus was very dense and showed telodendria with fine fibers extending between epithelial cells. Especially dense "end balls" of interdigitating arborizations were found in the subepithelial plexus of the pharyngoesophageal junction. She described no other structures of possible sensory significance.

Huber [16] considered the plexus of fine nerve fibers in smooth muscle to be exclusively motor. He did not think there were any sensory endings in this tissue.

Sabussow [29] studied the pharynx and esophagus of the dog, cat, and rabbit. Plexuses corresponding to those of Auerbach and Meissner were present as far forward as the dorsal wall of the pharynx. Sabussow [29] described cylindrical encapsulated end-bulbs of Krause in the pharynx, but could not find them in the esophagus. He agreed with DeWitt [9] that the sensory arborizations were in the subepithelial layer and that they sent fine fibers to form free endings between the epithelial cells.

Lawrentjew [20] studied the plexuses of the esophagus of the dog. He found the myenteric plexus throughout the esophagus, but the submucosal plexus was stained in the caudal third only.

A histological study of the nerves of the stomach wall in the sheep and goat [14] revealed several different kinds of nervous structures to which a sensory function might reasonably be attributed. A three-dimensional network of fine nerve processes surrounding the smooth muscle bundles in all parts of the stomach may be sensitive to pressure and tension. There is no reason to consider this network exclusively motor, as Huber [16] did. The terminal network contains tufts and skeins of anastomosing processes which may be sensitive. No encapsulated pressure receptors or tension spindles could be found. Long, undulating, nonmyelinated, single nerve fibers were seen in the connective tissue of the mucosa-submucosa. They often accompanied the capillaries and occasionally came into close apposition to the epithelium. Myelinated fibers were found in close proximity to the epithelium of the reticulum and the lips of the esophageal groove, but no specialized endings could be identified.

MATERIALS AND METHODS

The 50 sheep which were used in this series of experiments were mostly young western lambs weighing approximately 60 lb. However, since the work was continued over a nine-month period, the lambs which were used in the latter part of the work were relatively mature. A few mature and aged, locally-raised ewes were used in some of the experiments.

All of the experiments were acute and, in general, decerebration was done by the method described by Comline and Titchen.[8] Using ether anesthesia, the common carotid arteries were exposed and the skull was trephined, exposing a large part of the cerebral surface. The carotids were clamped during the sectioning of the sagittal sinus and during the actual removal of the cerebral mantle. Various methods were used to control hemorrhage—topical application of thromboplastic substance, bone wax, and ordinary mechanical methods. Best results were obtained using mechanical hemostasis, followed by gauze packing saturated with thromboplastic substance.

After decerebration, the clamps were removed from the carotids, and anesthesia was discontinued. The trachea was exposed and intubated to prevent the entrance of foreign material into the lungs and to permit forced artificial respiration. When it was necessary to observe the thoracic part of the esophagus, artificial respiration was applied, the left rib cage was removed, and left-lung pneumonectomy was performed. A positive pressure

apparatus was used in applying artificial respiration (fig. 1).

In most of the experiments, if a rumen fistula had not already been established, the rubber cannula was put in place after decerebration. This permitted intraruminal insufflation to stimulate eructation during the experiments. A number of modifications of this general procedure occurred throughout the work and will be described. Nitrogen was used as the intraruminal insufflating gas. Pressures were observed and recorded from an aneroid manometer and the rate of flow was measured with a rotameter.

Nerve stimulation was accomplished with a research model square wave stimulator * using shielded electrodes. Wave form and other characteristics of the electrical stimuli were evaluated with an oscilloscope.

Experimental results were recorded by cinematography. In some experiments, eructation was measured volumetrically with a gasometer. Because the nature of the work made it difficult to take notes while the experiments were in progress, a tape recorder was also used to record the results and observations.

Gross dissection of the nerves included both sides of 2 embalmed sheep and 1 embalmed cow. No gross anatomical variation was observed in dissecting the nerves for the physiological experiments. Frozen sections of the wall of the esophagus and cardia were impregnated with silver by the Bielschowsky-Gros method [21] to show the terminal distribution of the nerves.

* Stimulator made by Tektronix Inc., Portland, Ore.

RESULTS

Anatomy.—Removal of the jugular (paramastoid) process of the occipital bone, and reflection of the jugulohyoideus, digastricus, and rectus capitis lateralis muscles revealed cranial nerves IX, X, XI, and XII, as they emerged from the jugular foramen.

The glossopharyngeal nerve (IX) was the smallest and most rostral. It lay on the lateral surface of the cranial cervical sympathetic ganglion. One centimeter caudoventral to the bulla tympanica, it gave off the branch to the carotid sinus. At the same level, an anastomosis was detached to the vagus, and the glossopharyngeal nerve divided into lingual and pharyngeal branches. The lingual branch ran down the stylohyoid bone and passed deep to the hyopharyngeus muscle (m. constrictor pharyngis medius). The pharyngeal branch, at first caudal to the lingual, turned rostromesad deep to it, gave numerous branches to the hyopharyngeus, and passed over the dorsal surface of the stylopharyngeus. It was not traced farther.

Nerves X and XI were crossed laterally by the condyloid artery, which ran parallel and medial to the jugular (paramastoid) process. Nerve XI was dorsolateral, nerve

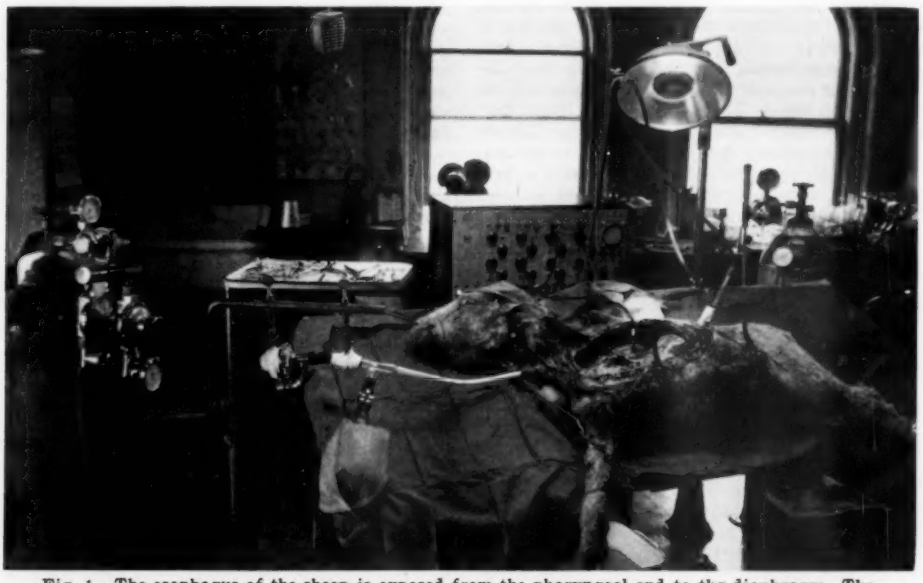


Fig. 1—The esophagus of the sheep is exposed from the pharyngeal end to the diaphragm. The artificial respiration and insufflating equipment and the stimulator are also shown.

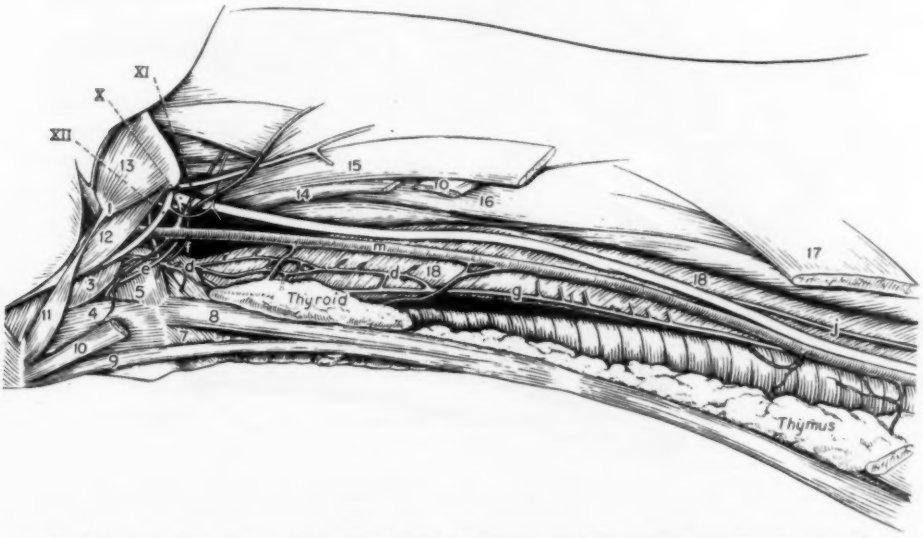
X was medial, and nerve XII ran between them as far as the tip of the jugular process; here, nerve XII turned ventrad, passing between X and XI, and over the lateral side of the condyloid artery. At the turn, XII gave off the ansa hypoglossi, which passed around the lateral side of the occipital artery to the first cervical nerve. Nerve XII then ran ventrorostrad along the caudal border of the digastricus muscle.

The vagus nerve (X) ran back medial to the occipital artery and became associated with the common carotid artery. Just before the vagus separated from the accessory nerve, it gave off the anastomosis to the glossopharyngeal nerve.

The next branch, also from the ventral edge of the vagus, was the pharyngoesophageal nerve (ramus pharyngeus, hominis). This ran ventrad, medial to the external carotid and ascending pharyngeal arteries, and turned caudad medial to the cranial laryngeal nerve. At the turn, it gave off a rostral branch distributed mainly to the chondropharyngeal part of the hyopharyngeus muscle.

The PEN continued caudad, receiving a large anastomosis from the cranial laryngeal nerve. This anastomosis was apparently the external laryngeal branch which, in the ruminant, runs with the pharyngoesophageal nerve to reach the cricothyroideus muscle.[3] Branches were given off to the thyropharyngeus and cricopharyngeus muscles (comprising the m. constrictor pharyngis inferior, hom.). Another branch, probably the continuation of the external laryngeal, innervated the cricothyroideus. A filament was traced into the thyroid gland.

In the sheep, a large anastomosis from the internal branch of the cranial laryngeal nerve emerged at the caudal border of the cricopharyngeus and joined the PEN. The latter was concealed on the ventrolateral surface of the esophagus by a thin layer of musculature. On the left side, the PEN could be traced to its anastomosis with the recurrent laryngeal nerve at the junction of the upper and middle thirds of the cervical esophagus. This point of anastomosis was also in the plane of the third cervical vertebra and the caudal end of the thyroid gland. On the right side, in the sheep, there was a direct end-to-end anastomosis with the dorsal or esophageal



(d) Pharyngoesophageal nerve; (e) cranial laryngeal nerve; (f) external laryngeal branch; (g) left recurrent laryngeal nerve; (j) cervical sympathetic trunk; (m) common carotid artery; (1) muscular angle of stylohyoid bone; (3) hyopharyngeus muscle; (4) thyrohyoid muscle; (5) thyropharyngeus muscle; (7) cricothyroid muscle; (8) sternothyroid muscle; (9) sternohyoid muscle; (10) omohyoid muscle; (11) stylohyoid muscle; (12) digastric muscle; (13) jugulohyoid muscle; (14) sternomastoid muscle; (15) cleidomastoid muscle; (16) longus capitis muscle; (17) scalenus muscle; (18) esophagus; (X, XI, XII) cranial nerves.

Fig. 2—The left side of the neck of the sheep.

branch of the recurrent nerve (to be described). A second thyroid branch was seen on both sides, entering the cranial third of the gland.

In the cow, the anastomosis from the internal branch of the cranial laryngeal nerve joined the recurrent nerve instead of the PEN. It is possible that this is an individual rather than a species difference.

Grossly, the upper third of the cervical esophagus appeared to be innervated by the PEN, rather than the recurrent nerve. According to Chauveau and Arloing,[3] the PEN, after its anastomosis with the recurrent nerve, can be traced to the lower part of the cervical esophagus in the horse and ruminant. The authors were unable to confirm this in dissections of the sheep and cow. After the anastomosis with the recurrent nerve, the PEN could not be traced farther down the esophagus by gross dissection. Its fibers may run caudad in the recurrent to reach the lower esophagus.

The cranial laryngeal nerve left the vagus medial to the origin of the occipital artery. The internal laryngeal branch passed ventrad with the laryngeal artery and disappeared in the triangle formed by

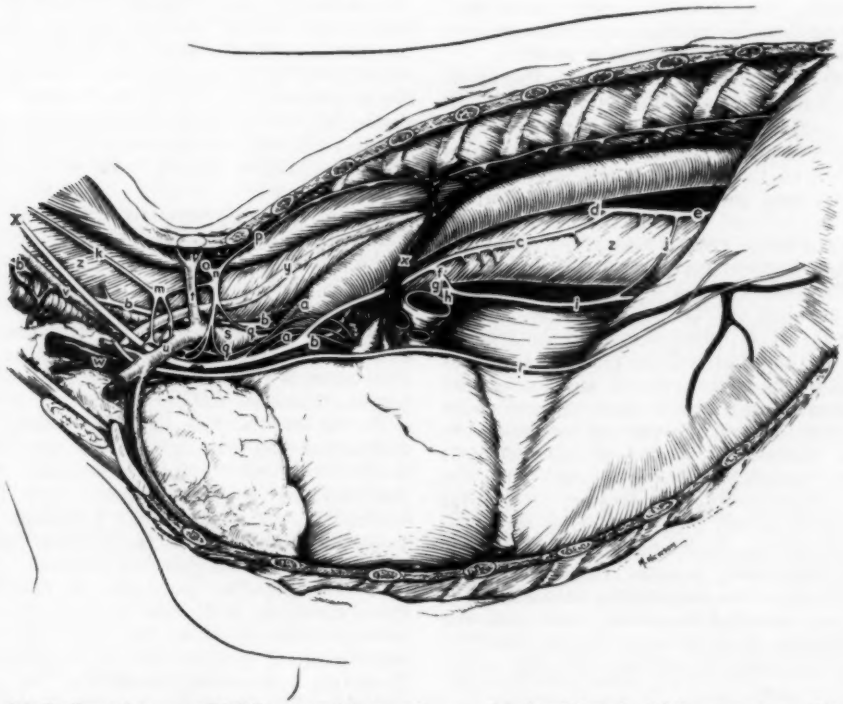
(a) Lingual branch of glossopharyngeal nerve; (b) pharyngeal branch of glossopharyngeal nerve; (c) branch to carotid sinus; (d) pharyngoesophageal nerve; (e) cranial laryngeal nerve; (f) external laryngeal branch; (g) left recurrent laryngeal nerve; (h) ansa hypoglossi; (i) cranial cervical sympathetic ganglion; (k) external carotid sympathetic plexus; (m) common carotid artery; (p) laryngeal artery; (q) ascending pharyngeal artery; (r) external carotid artery; (s) external maxillary artery; (t) lingual artery; (1) muscular angle of stylohyoid bone; (2) stylopharyngeus muscle; (3) hyopharyngus muscle; (4) thyrohyoid muscle; (5) thyropharyngeus muscle; (6) cricopharyngeus muscle; (7) cricothyroid muscle; (8) sternothyroid muscle; (9) sternohyoid muscle; (10) omohyoid muscle; (11) stylohyoid muscle; (IX, X, XI, XII) cranial nerves.

Fig. 3—The pharyngeal region after removal of the jugular (paramastoid) process and the jugulohyoideus, digastricus, and rectus capitis lateralis muscles.

the chondropharyngeus (rostrally), the thyropharyngeus (caudally), and the thyrohyoid (ventrally). The external laryngeal branch left the deep surface of the cranial laryngeal near the vagus and ran ventrad to anastomose with the PEN, as previously described. A large anastomosis from the internal laryngeal branch ran back inside the dorsal border of the thyroid cartilage, then across the deep surface of the cricopharyngeus muscle. At the caudal border of the muscle, it joined the PEN in the sheep, and the recurrent nerve in the cow. This was the same on right and left sides. According to Chauveau and Arloing,[3] the anastomosis is a motor nerve to the esophagus. Our stimulation of the central and peripheral ends of the cut cranial laryngeal nerve in several sheep indicated that it was sensory, not motor to the esophagus.

The inferior (nodose) ganglion was indicated only by a slight enlargement of the vagus in the region of the origin of the cranial laryngeal nerve. Histological sections revealed numerous ganglion cells at this point. The sympathetic trunk joined the vagus on the medial side at this level, and the two were united in the carotid sheath. On the left side, the sympathetic trunk separated from the vagus at the fifth cervical vertebra and took a more dorsal course on the esophagus. On the right, the sympathetic separated from the vagus at the third cervical vertebra, and ran dorsal to the carotid on the trachea.

On the left, the vagus passed medial to the subclavian artery and costocervical



(a) Cardiovagal nerves; (b) left recurrent laryngeal nerve; (c) dorsal branch of left vagus nerve; (d) dorsal branch of right vagus nerve; (e) dorsal vagal trunk; (f) ventral branch of left vagus nerve; (g) ventral branch of right vagus nerve; (h) bronchial branches; (i) ventral vagal trunk; (j) anastomosis (variable); (k) cervical sympathetic trunk; (m) middle cervical sympathetic ganglion; (n) cervicothoracic sympathetic ganglion; (o) vertebral nerve; (p) thoracic sympathetic trunk; (q) cardiosympathetic nerves; (r) phrenic nerve (roots removed); (s) common brachiocephalic trunk; (t) costocervical trunk; (u) left subclavian artery; (v) common carotid artery; (w) jugular vein; (x) azygos vein; (y) thoracic duct; (z) esophagus; (X) cervical vagus.

Fig. 4—Nerves in the thorax of the sheep.

vein, along the ventrolateral surface of the common brachiocephalic trunk, and curved dorsocaudad across the left side of the aortic arch. A branch was detached to the cranial surface of the arch, another branch entered the cardiac plexus caudal to the arch, and the recurrent laryngeal nerve turned mesad around the caudal side of the arch.

The left recurrent nerve ran craniad on the ventral surface of the esophagus, where it could be seen by depressing the common brachiocephalic trunk. In the neck, it coursed medial to the common carotid artery in the groove between the esophagus and the trachea. The caudal two thirds of the cervical esophagus received numerous branches from the recurrent nerve. A few branches went to the trachea. After the anastomosis with the PEN (previously described), the recurrent nerve continued craniad on the trachea and disappeared deep to the cricopharyngeus muscle.

The right recurrent nerve left the vagus caudal to the subclavian artery. It crossed the medial surface of the vertebral and costocervical arteries, crossed the dorsal surface of the right common carotid, and ran craniad, medial to the carotid, on the ventral surface of the trachea. In the sheep, a large branch was given off at the seventh cervical vertebra. It passed dorsad between the trachea and the carotid, and ran craniad on the dorsal surface of the trachea. It supplied nerves to the trachea and, in the middle of the neck, its branches reached the right ventral surface of the esophagus and ran in both directions in the musculature. Continuing up the esophagus, this dorsal branch of the recurrent nerve anastomosed end-to-end with the PEN. The recurrent proper, or caudal laryngeal nerve, inclined dorsad from the middle of the neck and passed under the cricopharyngeus muscle. It gave no more branches to the esophagus, cranial to the seventh cervical vertebra, and did not anastomose with the PEN. In the cow, the right recurrent nerve was a single trunk like the left.

The left vagus, after giving off the recurrent nerve, passed between the aorta and the vena azygos. Caudal to the aortic arch, between the esophagus and the left bronchus, it divided into dorsal and ventral branches.

The right vagus ascended steeply behind the subclavian artery, between the costocervical vein and the trachea, and over the apical bronchus. Numerous branches were given off to the top of the trachea, and a few to the esophagus. At the fourth rib, dorsal and slightly cranial to the right bronchus, the right vagus divided into dorsal and ventral branches.

The right and left dorsal branches inclined to the dorsal surface of the esophagus and united at the level of the eighth or ninth thoracic vertebra to form the dorsal vagal trunk, which passed through the diaphragm. The dorsal branches and the dorsal trunk supplied twigs to the esophagus.

The right and left ventral branches gave off the bronchial branches and united immediately, just behind the bifurcation of the trachea. The ventral vagal trunk so formed ran in the mediastium ventral to the esophagus and slightly to the left of the median plane. It was not so closely associated with the esophagus as the dorsal trunk, and no twigs were seen passing to the esophagus from the ventral trunk. Occasionally, an anastomosis connected dorsal and ventral trunks across the left side of the esophagus just in front of the diaphragm.

In the silver-impregnated sections of the wall of the esophagus from the midcervical region, nerve processes in close apposition to the epithelium were rare, although the terminal nervous network in the adjacent muscularis mucosa was well impregnated, and nerves were readily demonstrated in association with the arterioles in the lamina propria mucosae.

At the cardia, fine nerve processes accompanied the capillaries into the long narrow connective tissue papillae that reach a level only 40 to 50 μ below the free surface of the epithelium. These microscopic papillae belong to the stratum papillare of the lamina propria mucosae. Because the papillae penetrate so far into the epithelium, it is easy to gain the impression from vertical sections that the accompanying nerves are intraepithelial. However, sections cut parallel to the epithelial surface were also examined and no evidence could be found that the nerves actually leave the connective tissue and penetrate between the epithelial cells.

A ganglionated myenteric plexus was demonstrated in sections from the cardia, midcervical region, and cranial end of the esophagus. The motor end plates of the striated muscle were also shown.

MOTOR INNERVATION

Stimulation Experiments.—In this series of experiments involving 20 sheep, the rumen and reticulum were insufflated with nitrogen gas, using pressures varying between 15 mm. and 35 mm. Hg. Gas was insufflated at rates varying between 3 and 6 liters per minute. Stimulation of the nerves while the esophagus was dilated with gas or during the dilation phase of eructation, made it possible to evaluate critically the limits of the motor nerve influence. In most cases, the intact nerves were stimulated but, in 2 sheep in this series, the nerves were cut and the peripheral ends were stimulated.

Stimulation of the PEN caused contraction of the cranial sphincter[11] of the esophagus, and of the cervical part of the esophagus as far caudally as the midcervical region and, in the majority of cases, down to and even caudal to the thoracic inlet.

Stimulation of the left recurrent nerve at the aortic arch usually caused contraction of that segment of the esophagus extending from the region dorsal to the heart as far craniad as the thoracic inlet. However, in a few cases the motor activity extended well toward the cranial end of the esophagus. In 2 sheep, stimuli applied to the left recurrent nerve had little noticeable effect on any part of the esophagus.

Stimulation of the dorsal vagal trunk caused contraction of the esophagus from the region of the heart to the stomach. This was constant, although, in a few animals, the motor activity extended almost to the thoracic inlet. In one sheep the right recurrent nerve was stimulated just cranial to the thoracic inlet. Electrical stimuli of 20 to 50 v., frequency of 50 per second, and a pulse duration of 5 msec. were used. Contraction of the esophagus occurred to the midcervical region, but there was no response caudal to the thoracic inlet. In another sheep, stimulation of the right recurrent nerve cranial to the thoracic inlet caused minor twitching of the esophagus up to the laryngeal region.

Report on One Experiment.—One experiment will be described in detail. A sheep was decerebrated in the usual manner. The entire esophagus was exposed. Stimulation was set for 2.5 seconds' duration, with a cyclic rate of 50 per second, a pulse width of 5 msec., and a voltage of 25.

I) *Intact Nerves.*—The results of stimulation of intact nerves were:

1) Left PEN—good contraction throughout the cervical esophagus extending into the thoracic inlet, followed by swallowing movements after removal of the stimulus.

2) Right PEN—same response as left PEN.

3) Left vagus in midcervical region—contraction of the thoracic esophagus extending to midcervical region.

4) Right vagus in midcervical region—about the same action as when left vagus was stimulated.

5) Left recurrent before circling the aorta—contraction of esophagus from 5 cm. cranial to the thoracic inlet to the diaphragm.

6) Left recurrent after circling the aorta—about the same as 5, but more a twitch than a well-defined contraction.

7) Dorsal thoracic vagus—strong contraction in the thoracic portion of the esophagus. No noticeable contraction of esophagus cranial to the thoracic inlet.

8) Ventral thoracic vagus—clonic type of contractions of esophagus cranial to the diaphragm.

9) Right recurrent nerve just cranial to the thoracic inlet—contraction of esophagus to midcervical region. No response caudal to thoracic inlet.

10) Left recurrent nerve cranial to the thoracic inlet—slight contraction of esophagus cranial to the thoracic inlet.

11) Left cranial laryngeal—repeated swallowing.

12) Right cranial laryngeal—repeated swallowing.

II) *Sectioned Nerves.*—The results of stimulation of sectioned nerves were:

1) Section of left vagus (midcervical), all other nerves intact: (*a*) stimulation of peripheral end—contraction of esophagus in thoracic portion, no visible contraction cranial to thoracic inlet, strong rumen contraction; (*b*) stimulation of central end of sectioned left vagus—swallowing and peristaltic waves going down the esophagus.

2) Section of right vagus in midcervical region: (*a*) stimulation of peripheral end—contraction of esophagus in the thoracic region back to the diaphragm; (*b*) central end—no effect noticed; (*c*) stimulation of left PEN—contraction of esophagus back to the thoracic inlet; (*d*) left cranial laryngeal—swallowing movements; (*e*) left recurrent nerve before rounding the aorta—slight contraction dorsal to the heart.

III) *Left* PEN.—Results of stimulation after section of the left PEN were:

1) Peripheral end—contraction of the esophagus to the thoracic inlet.

2) Central end—pharyngeal contractions.

IV) *Right* PEN.—Results of stimulation after section of the right PEN were:

1) Peripheral end—contraction of esophagus to thoracic inlet.

2) Left cranial laryngeal—laryngeal movements, but no swallowing.

3) Left recurrent nerve cranial to thoracic inlet—some laryngeal movements.

4) Ventral thoracic vagus—a number of stimuli caused tetanic contractions of the esophagus.

5) Dorsal thoracic vagus—contraction of esophagus to the diaphragm.

In this series of experiments, considerable variation was observed in the limits of motor activity during nerve stimulation. There was also a tendency for overlapping of the motor influences of each of the three pairs of nerves supplying the esophagus: the PEN, the recurrent, and the thoracic vagus.

Effects of Curare.—A decerebrate sheep was prepared and the esophagus was exposed from the cranial end to the diaphragm. Electrodes were placed on the motor nerves of the esophagus. The left sciatic nerve was exposed and an electrode was applied to it. The efficiency of eructation was tested and the motor nerves of the esophagus were stimulated. After photographically recording the visual motor events, curare was injected (12 mg. tubocurarine chloride) until electrical stimulation of the sciatic nerve no longer elicited the leg kick. Electrical stimuli applied to the motor nerves of the esophagus were without effect. The curare effect terminated at or near the cardia.

Diaphragmatic Motility.—In the course of the experiments, stimulation of the dorsal vagus sometimes caused a contraction of the crura of the diaphragm. Dissection of fresh and fixed specimens did not reveal any branches from the dorsal or ventral vagi to the crura in the region of the hiatus esophageus; yet, when the phrenic and vagus nerves were sectioned and the crura were separated from the sublumbar region, or when the cervical cord was sectioned, stimulation of the distal end of the cut dorsal vagus caused a contraction of the crura of the diaphragm. These motor phenomena were noticed in about half the animals in which this particular motor event was under observation. The shielded electrodes employed should have prevented electrical stimuli from spreading to the other tissues.

In one experiment, after the brain stem was destroyed, the esophagus relaxed and gas passed through it at a more or less constant rate. There were no normal eructation movements.

In another experiment, artificial respiration was instituted and the cord was cut at the atlanto-occipital junction. Eructation was similar to that seen in other decerebrate preparations.

EXPERIMENTS ON AFFERENT NERVES AND RECEPTORS

Experiments were designed to study the afferent pathways and the receptors involved in eructation.

The Right Ruminal Nerve.—The vagal branches in the right longitudinal groove were cut at successively higher levels from the caudal end to a point two thirds of the distance from the caudal end to the cardia. This had no noticeable effect on eructation in the decerebrate sheep.

The Effects of Body Position on Eructation.—In this series of experiments, the effects of gravity on the contents of the rumen and reticulum and the subsequent effects on eructation were studied. Sheep with normally filled rumens were decerebrated and a rumen fistula cannula was put in place. They were then strapped on a table that could be tipped laterally 45 degrees in either direction. Insufflation at a given rate was started with the sheep in lateral recumbency. The sheep were then placed at 45 degrees in the sternal position and, finally, at 45 degrees with the dorsum down. The rumen was insufflated at constant rates for several minutes while the sheep were in each of the above positions.

Eructation was more efficient in the sternal position than in lateral recumbency. With the dorsum down, eructation was completely inhibited. The rumen and reticulum were emptied of all contents and again the rumen was insufflated with the sheep in the three positions. Eructation was equally efficient in all positions. Approximately 2,500 ml. of warm tap water was placed in the empty rumen. The initial picture was again seen. Eructation was more efficient in the sternal position than in lateral recumbency and was inhibited with the dorsum down.

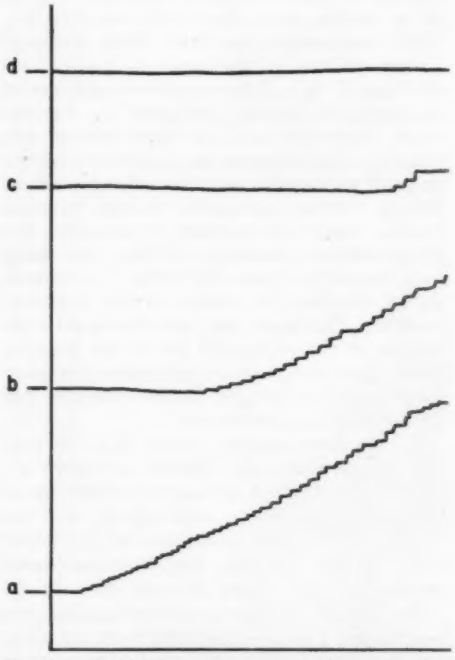
Ablation Experiments.—The previous experiments indicated that some of the receptors inhibiting eructation, as well as those stimulating it, might be located around the cardia. Decerebrate sheep were prepared in the usual manner. The abdominal cavity was opened and large stomach clamps were applied in such a manner as to leave only a relatively small pocket of the rumen near the cardia and, possibly, a small section of the dorsal part of the reticulum. All of the rumen and reticulum distal to the clamps was removed. A

small cannula was placed in the remnant (cardia pouch). Insufflation into the pouch elicited eructation. A small amount of water (100 ml.) inhibited eructation. Removing the water restored eructation to its initial cardia pouch efficiency. An injection of 100 ml. of mineral oil also inhibited eructation.

Measuring Eructation Quantitatively Under Various Conditions.—Four sheep were included in this series of experiments. The sheep were decerebrated in the usual manner and only the midcervical trachea was exposed. A cannula with cuff was placed in the trachea caudal to the tracheal incision to allow unimpeded breathing. Another tracheal cannula with cuff was directed toward the larynx. The end of this cannula was connected by rubber tubing to a trap common to a tube coming from an airtight mask. From the side-arm-flask trap, eructated gas was passed through a one-way valve to a large gasometer and, through a special mechanism, the volumes of gas eructated were recorded on the drum of a small spirometer. The rumen and reticulum were emptied from an incision in the caudal surface of the dorsal blind sac. This incision was then closed (gastight) with an 8-inch hernia clamp. A clamp was tightened on the neck of the omasum, confining the insufflated gas to the first three compartments of the stomach. A rumen cannula was then put in place, and the abdominal wall sutured or temporarily closed with tissue forceps.

The inhibitory effects of the varying amounts of water in the rumen and reticulum, when the sheep were placed in 30 degrees dorsal recumbency, are shown (graph 1).

Mucous Membrane Treatment with Butyn Sulfate.—The above experiments indicated that sufficient receptors were present around the cardia to initiate reflexes exciting or inhibiting eructation. This suggested another series of experiments, using 6 sheep, directed at blocking one or both of these groups of receptors. The sheep were decerebrated; the rumen and reticulum were cleared of ingesta and wiped out with gauze packs. A cannula was placed in the rumen and the incision was closed tightly with a large hernia clamp. The body wall was closed with tissue clamps. Nitrogen was insufflated with the sheep in dorsal recumbency at about 30 degrees from the vertical position. This produced active eructation. In the first preparation (a small sheep), 100 ml. of water inhibited eructation completely. The water was removed from the rumen and reticulum, and 100 ml. of 1 per cent butyn sulfate solution was placed in the rumen and reticulum. After a lapse of three minutes, nitrogen was insufflated at 3 liters per minute with the sheep again in dorsal recumbency (30 degrees from the vertical position). The intraruminal pressure only rose to 8 or 9 mm. Hg before fluid started coming out of the mouth, nostrils, and the cranial end of the incised trachea. Eructation movements seemed to be active. Of the other sheep, 3 responded in a similar manner; however, it was necessary after butyn sulfate application to insufflate to higher pressures before fluid passed the cranial sphincter freely. The neck of the omasum was clamped in these 3 sheep, limiting insufflated gas to the first two compartments. Removal of the clamp in 2 of these sheep and insufflation after 1 liter of water was added caused a mixture of water and gas to be eructated at pressures



Graph 1—Eructation measurement. Rumen insufflation with sheep 30 degrees dorsal recumbent—(a) rumen empty; (b) 500 ml. water in rumen; (c) 1 liter of water in rumen; (d) 1,500 ml. water in rumen.

varying between 10 and 15 mm. Hg. With the clamp on the neck of the omasum again, it was necessary to increase the intraruminal pressures to 25 to 30 mm. Hg before the cranial sphincter gave way to a mixture of fluids and gases.

At the conclusion of the final experiment, in which the mucosa was anesthetized with butyn sulfate, the PEN was isolated and stimulated at 30 v., cyclic rate of 40 per second, and a pulse width of 5 msec., for several minutes. This stimulation was continued during nitrogen insufflation at 6 liters per minute. At first, the cervical part of the esophagus remained contracted back to the thoracic inlet. At about 90 mm. Hg pressure, the cervical esophagus began to distend to the cranial esophageal sphincter. At 125 mm. Hg, the abomasum was suddenly distended and the pressure was discontinued.

Esophageal Pressures During Eructation.—Only 1 sheep was used in this experiment. This sheep had a permanent rumen cannula fistula. Under ether anesthesia, a short piece of 3-mm. polyethylene tubing was passed through a small incision in the esophagus in the midcervical region. The intraesophageal end was directed downward toward the cardia for a distance of 5 to 7 cm. After a few unsuccessful attempts to record pressure in various ways, the free end of the tubing was coupled to an aneroid manometer. Another aneroid manometer in the nitrogen insufflating tubing, connected to the cannula fistula, made it possible to measure the intraruminal pressure. After the sheep had recovered from the ether anesthesia, it was confined in stocks in the standing position. Nitrogen was insufflated into the rumen at the rate of 5 liters per minute. Since this was only a preliminary experiment, only a few general statements will be included in this report.

In the lower ranges, from 10 to 35 mm. Hg of pressure, the rumen pressure exceeded the highest pressures occurring in the esophagus during eructation. For example, when the intraruminal pressures were 10 and 15 mm. Hg, the esophageal pressures were 5 and 10 mm. Hg.

At higher intraruminal pressures, the esophageal pressures tended to be equal or nearly equal to the intraruminal pressures.

When 9 liters of water were placed in the rumen, and the back of the retaining crate was raised 1 foot, eructations were inhibited, and intraesophageal pressures exceeded intraruminal pressures. For instance, at an intraruminal pressure of 46 mm. Hg, the pressure in the esophagus rose to 60 mm. Hg and the animal began to collapse.

DISCUSSION

The decerebrate animals were excellent subjects for the studies herein reported. Although, in many cases, the surgery was extremely drastic, it was felt that surgical shock had little bearing on the results obtained. The statement is based on the observed acuteness of the reflexes, heart action, and general vitality of the experimental animal.

In the studies of the motor nerve supply to the esophagus, considerable variation was observed in the extent of the parts supplied by the different vagal branches. This could have been caused, in part, by differences in dissection techniques, in which the degree of injury to the nerve and blood supply to the esophagus was unavoidably varied. However, the main variations were thought to be due to individual anatomical and physiological differences. The greatest variation in esophageal motor response occurred when the recurrent nerves were stimulated. Throughout this part of the work, it seemed that the region supplied by each nerve complemented that of the other nerves. There was an overlapping of the esophageal areas supplied by different nerves in over half of the prepared animals.

It is felt that the physiological demonstration of receptors around the cardia, capable of initiating reflexes that stimulate and inhibit eructation, is one of the most important contributions of this work. This does not imply that such receptors occur only around the cardia; they may be widely distributed in the walls of the rumen and reticulum and even in the esophagus.

Under the conditions of the experiments, it was demonstrated that sufficient receptors are present in a relatively small area around the cardia to initiate the reflexes which stimulate or inhibit eructation. No definite evidence is presented to characterize the receptors as to type. Eructation is stimulated by a variety of gases and is inhibited by water, mineral oil, or fluid ingesta. It may be that the inhibitory mechanism is dominant and that eructation is stimulated by the stretch receptors demonstrated, physiologically, by Iggo [19] and represented, morphologically, by the

terminal nervous network in the wall of the stomach.[14] Possibly, the inhibitory receptors are the fine nerves that accompany the vascular connective tissue papillae up into the epithelium.

The observation that the eructation reflex could be stimulated by gas pressure, when the eructation inhibitory reflex was blocked by local application of butyn sulfate, suggests that the stimulatory receptors are deeper than the inhibitory receptors.

The return of the eructation inhibition mechanism to nearly normal activity when the clamp was placed on the neck of the omasum suggests, at least, that this method of confining gas, fluids, or both, to the first two compartments of the stomach is not physiologically sound, at least for the butyn sulfate experiments. Much thought has been devoted to improving methods of closing the reticulo-omasal orifice with a minimum of disturbance to the rich nerve supply in this region. The efforts so far have been disappointing. Because of the relative inactivity of the reticulum and rumen in decerebrate preparations, the reticulo-omasal orifice must remain more or less closed. Certainly no large amounts of gas, water, or both, have been found in the abomasum or intestine until high (over 60 mm. Hg) intraruminal pressures have been attained. Although our experimental procedure has some decided weaknesses, careful observation indicates that only small amounts of the butyn sulfate passed beyond the reticulo-omasal orifice.

It has been shown in previous work [11] that eructation can be inhibited pharmacologically. The inhibition may be partial or complete. The work herein reported shows that eructation can be inhibited partially or completely by flooding the cardia and the region around it with water, ingesta, or both. Just what part either method of inhibition plays in bloat is a question of utmost importance.

The difference in the highest intraesophageal pressure attained during eructation and the intraruminal pressure indicates that, at low or moderate intraruminal pressures, the cardia permits only a certain amount of gas to enter the esophagus. At higher intraruminal pressures, and under conditions when fluids can not be prevented from entering the cardia, the higher intraesophageal pressures are probably caused by strong deglutitory movements with a closed cranial esophageal sphincter.

Another observation of importance is the demonstration of partial or complete inhibition of eructation by creating a situation that made clearing of ingesta or water from the cardia inefficient or impossible. One of us (R.W.D.) has observed a large number of bloated animals on pasture. Eructation is usually not completely inhibited until the animal has reached the distress stage, when it may be completely inhibited. In the early stages, there might well be a considerable reduction in eructation efficiency resulting from a partial inhibition. Johns [20] stated that neither rumen movement nor the eructation reflex were inhibited during the onset of bloat. Since no records can be found of quantitative measurement of eructation in ordinary bloat cases, it would seem that the information here reported is of utmost importance if partial inhibition of the eructation reflexes is of real significance in the onset of bloat.

Summary

1) The results herein reported are from acute experiments on decerebrate sheep.

2) A detailed study was made of the anatomical structures involved in eructation.

3) The pharyngoesophageal nerves supplied motor innervation to the cranial esophageal sphincter and to the cervical part of the esophagus down to the mid-cervical region in some animals, and to the thoracic inlet in others.

4) The recurrent laryngeal nerves supplied the motor innervation to the cranial thoracic part of the esophagus and, in some sheep, also to the cervical part. The action obtained from stimulation of the recurrent nerve was variable.

5) Stimulation of the dorsal branch of the thoracic vagus caused contraction of the esophagus from the midthoracic region, to and including the cardia.

6) In general, the extent of esophageal motor activity produced by stimulating any one of the three pairs of nerves was variable. The three pairs of nerves functioned together as a unit, and the variation of one pair was compensated for by a corresponding variation in the others.

7) Receptors have been demonstrated in a relatively small area around the cardia which, when properly stimulated, will cause reflex excitation or inhibition of eructation.

8) Inhibition was caused by flooding the cardia with water, ingesta, or mineral oil in such a manner as to embarrass mechanically the normal cardia-clearing mechanism.

9) Eructation was stimulated by insufflating nitrogen into the intact rumen or into a small pouch formed by the walls of the rumen and reticulum adjacent to the cardia.

10) Sectioning the nerves in the right longitudinal groove of the rumen at various levels did not inhibit eructation.

11) When the rumen and reticulum were left intact and made gastight after removing all ingesta and liquids from both, and when the sheep was tilted dorsum down, varying amounts of water placed in the rumen and reticulum either reduced the efficiency of eructation or completely inhibited it.

12) The cranial sphincter was the most effective of the esophageal sphincters in preventing eructation when the inhibitory reflex was stimulated.

13) A 1 per cent butyn sulfate solution, placed in the cranial part of the rumen and in the reticulum, blocked the eructation inhibitory influence of water covering the cardia.

14) In an experiment in 1 sheep, the cardia seemed to regulate gas passing into the esophagus from the rumen. At lower pressures, the intraruminal pressures exceeded those of the esophagus during eructation. At higher pressures, with a fluid-embarrassed cardia, the magnitude of the pressures was reversed.

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Effectiveness of Dow ET-57 (Trolene)* Against the Nasal Botfly of Sheep

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LARVAE of the nasal botfly, *Oestrus ovis*, are species-specific parasites of sheep which are located in the frontal sinuses and nasal cavities. Infected sheep usually harbor all stages, *i.e.*, the first, second, and third instars. First instars range from about 2 to 5 mm. in length, and are most frequently found embedded in the mucus covering the mucous membranes of the nasal septums, turbinate bones, and nasal meatuses. Second instars range from 5 to 12 mm. in length, and are usually found in the posterior part of the nasal cavity, the ethmoid region, and often in the frontal sinuses. Third instars are between 12 and 25 mm. in length and are approximately 8 mm. in diameter through the thickest part. They inhabit the frontal sinuses but may also be found in the nasal cavity while in the process of emerging.

The nasal botfly of sheep is widely distributed throughout the world. In New Mexico, where this investigation was carried out, from 90 to 95 per cent of adult sheep are probably infected at some time. The incidence is somewhat lower among lambs than among adult animals.

In 1940, Cobbett [1,2] described a treatment whereby the nasal cavity was infused with a 3 per cent saponated cresol solution. Administration required the restraining of individual sheep, on their backs, upon a table or bench having a V-shaped surface. The saponated cresol solution was kept under pressure in a steel tank to which was attached a hose and a nozzle which was adapted for insertion into the nostrils. This method of treatment is used in New Mexico and, when properly applied, destroys 95 to 98 per cent of the first instars.

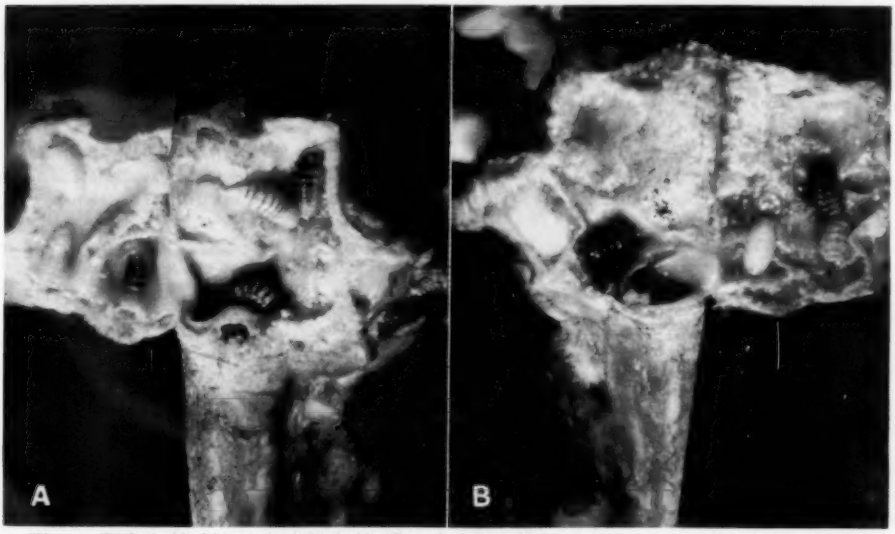

Fig. 1—Left half (A) and right half (B) of a sheep's head with the frontal sinus opened, showing third instar larvae of Oestrus ovis.

The purpose of this paper is to report a

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* Supplied by the Agricultural Chemical Development Division, Dow Chemical Co., Lake Jackson, Texas.

new method of treatment for the control of *O. ovis*, using as a drench the organic phosphate insecticide 0,0-dimethyl 0-2,4,5-trichlorophenyl phosphorothioate, more commonly known as Dow ET-57 or Trolene.

MATERIALS AND METHODS

The 56 sheep used were aged ewes from an open range in New Mexico and were kept in a drylot at the laboratory. They were allotted to two groups of 28 each, weighed, and individually identified with ear tags. A 50 per cent emulsifiable concentration of the drug (av. dose 9.2 ml.) was administered to the sheep of one group with a dose syringe, without the addition of water. The daily food ration was not reduced before treatment. The quantity of active ingredient administered to each animal was equivalent to 100 mg. per kilogram of body weight. The remaining 28 sheep served as untreated controls.

The drug was given at different times, and an equal number of treated and untreated sheep were killed between 72 and 96 hours after treatment (not more than 14 per day), and examined for the presence of larvae. Thus, the principal and control groups were divided into five groups (table 1) and all were treated or examined during December.

To expose the nasal cavity for examination, each head was split sagitally, and all larvae on the entire surface of the septum, the nasal turbinates, meatuses, and ethmoid processes were counted. The frontal sinuses were exposed by sawing in a frontal plane from the supraorbital process to the median plane (fig. 1).

RESULTS

An evaluation of the results of the treatment was made by comparing the number of larvae which were found in treated and untreated sheep (table 1).

A single drench with a dose of 100 mg. per kilogram of body weight destroyed all first instars in all but 1 of the 28 sheep treated. This animal harbored 116 first instars, the only ones which were found in the treated sheep, as compared with 1,769 which were in the 28 untreated control animals. The morning following treatment, this same animal was suffering from profuse diarrhea. Since the other 27 treated sheep exhibited no diarrhea, the condition was not attributed to the treatment.

In the treated sheep, 2 dead and 79 live second instars were found, as compared with 2 dead and 132 live second instars found in the control animals.

In the treated sheep, there were 19 dead and 15 living third instars, as compared with 18 dead and 7 living in the untreated controls. There apparently was little effectiveness against the larvae in the second and third instar stages. The total number of living and dead larvae in all three stages of development in the treated sheep was 232, as compared with 1,928 in the untreated controls, which indicates that the total infection was reduced about 89 per cent as a result of a single treatment.

DISCUSSION

This drug was most effective against first instars; therefore, the treatment, for best results, should be administered in the late

TABLE 1—Results Obtained with Dow ET-57 (Trolene), 50 per Cent Emulsifiable Concentration, Administered as a Drench to Sheep Infected with Larvae of Oestrus Ovis *

Group	Date of necropsy	No. of sheep	No. infected	Per cent infected	First instars		Second instars		Third instars	
					Dead	Alive	Dead	Alive	Dead	Alive
I										
Principal	12/11/56	5	2	40.0	0	116†	0	11	0	1
Control		5	5	100	0	337	0	31	5	2
II										
Principal	12/14/56	4	3	75.0	0	0	0	14	9	7
Control		4	4	100	0	406	0	18	4	0
III										
Principal	12/21/56	7	6	85.7	1	0	2	22	1	4
Control		7	7	100	0	304	2	12	4	0
IV										
Principal	12/22/56	5	3	60.0	0	0	0	11	1	0
Control		5	5	100	0	322	0	17	1	0
V										
Principal	12/31/56	7	7	100	0	0	0	21	8	3
Control		7	7	100	0	360	0	54	4	5
Totals										
Principal		28	21	75.0	1	116	2	79	19	15
Control		28	28	100	0	1,769	2	132	18	7

* Dosage = 100 mg. per kilogram of body weight; interval between treatment and examination = 72 to 96 hours.

† The 116 first instars found in this group were all from one sheep. This animal was observed to have profuse diarrhea the morning following treatment.

fall and early winter. In New Mexico, first instars are found in infected sheep throughout the summer but, because of continuous reinfection occurring from late spring to early fall, it is advisable to delay treatment until after the fly season, which usually terminates with the first hard frost.

The treatment was 100 per cent effective against first instars in all but 1 animal. This particular animal was found to be troubled with diarrhea which could not be attributed to the treatment. Since various types of diarrhea are common in range and feedlot sheep, additional data on the effectiveness of this drug in the presence of diarrhea are needed.

During the examination of the sheep following treatment, it was observed that the blood supply to the nasal cavity, which is inhabited by first instars, was much richer than the supply to the ethmoid region and frontal sinuses, which are occupied by the second and third instars. The first instars were, therefore, more closely associated with the blood stream carrying the systemic insecticide and, for this reason, it was thought they were exposed to a dosage greater in proportion to their size than were the second or third instars. However, the first instars may be more vulnerable.

Sheep tolerate this drug well, and probably would endure considerably larger doses than employed in these tests. It may be possible to increase the effectiveness by increasing the dose.

While this treatment is not recommended for general use as a systemic agent against sheep nose bots, on the basis of the data obtained thus far, it does appear to offer advantages (simplicity, efficiency, and safety) that recommend its provisional trial by veterinarians who would otherwise be obliged to resort to topical medication with saponated cresol, tetrachloroethylene, or lindane.

SUMMARY AND CONCLUSIONS

Twenty-eight aged ewes, which were naturally infected with *Oestrus ovis*, were given a single treatment of 0, 0-dimethyl 0-2, 4, 5-trichlorophenyl phosphorothioate (Dow ET-57, or Trolene) as a 50 per cent emulsifiable concentration, by drenching at a dosage of 100 mg. per kilogram of body weight. Three to four days after treatment, all the first instars, except those in 1 of the 28 animals (93.5%) treated, were found dead. At the dosage level used, the treatment was ineffective against the second and third instars. The drug was well tolerated by the experimental animals, and no ill effects attributed to the treatment were observed.

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A Comparison of Reconstituted Skim Milk and Egg Yolk-Sodium Citrate as Extenders for Ram Semen

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THE POSSIBILITY of using boiled milk as an extender for ram semen was first suggested in 1949 by Mikhailov.[3] Dauzier *et al.*[1] found that ram semen was better preserved in egg yolk-sodium citrate extender than it was in boiled skim milk. If reconstituted skim milk could be used as an extender for ram semen, it would be more economical and more easily prepared than other extenders. Skim milk would also provide an excellent medium for the microscopic examination of semen. The purpose of this investigation was to compare reconstituted skim milk and egg yolk-sodium citrate as extenders for ram semen.

MATERIALS AND METHODS

The semen used in these studies was collected from 4 yearling Suffolk rams that were ejaculated twice weekly over a four-week period during October and November, 1955. The rams were allowed to mount restrained ewes and the ejaculum was caught in an artificial vagina. Following collection, the semen was split equally into each extender at a dilution of 1:100. The temperature of the extenders at the time of dilution was 80 to 90 F. The samples were allowed to cool in water at room temperature and then were placed in a refrigerator operating at 38 F. for storage studies.

Three skim milk powders, Borden (B), Carnation (C), and Pet (P), were used for reconstituting purposes. Powders B and C were reconstituted by adding sufficient distilled water to 22.3 Gm. of powder to form 250 cc. of skim milk. Powder P was reconstituted by adding sufficient distilled water to 24.3 Gm. of powder to form 250 cc. of skim milk. The C and P milks were heated at 92 to 95 C. for five minutes to destroy lactenin, identified by Flipse *et al.*[2] as a spermicidal factor in milk. The B milk was not heated because preliminary data at this station had shown that unheated B reconstituted skim milk would maintain the life of bovine sperm cells for extended periods. All milk samples were cooled to 38 F. and antibiotics were added as follows: streptomycin sulfate (1,000 μg/cc. of extender), and potassium penicillin G (1,000 units/cc. of extender). The milk extenders were stored at 38 F. for one or two days prior to being used.

The egg yolk-sodium citrate extender was composed of 20 per cent egg yolk and 80 per cent 0.1 M sodium citrate. The antibiotics were added in the same concentrations as in the skim milk.

Estimations of spermatozoa livability were made by microscopic readings (300 x) of extended semen immediately after collection, one day following collection, and thereafter on alternate days for the 15-day storage period. All readings were made with a microscope-stage incubator operating at 102 F.

RESULTS

The results of this experiment are shown (graphs 1–4). The semen motility readings plotted on the graphs are the ratings of all collections for each ram on each reading day.

The trend of sperm cell survival was approximately the same for each ram in all four extenders. In general, the skim milk samples appeared to maintain sperm cell life somewhat better than the egg yolk-sodium citrate. The P milk appeared better than the other kinds of reconstituted skim milk.

DISCUSSION

IT is recognized that, at this time, the artificial insemination of sheep is of little practical value in the United States. It may have a greater use in the future since it would promote the more widespread use of superior rams. If used in sheep, many problems would need to be solved, just as in the artificial insemination of cattle. One of these problems would be to develop a simple and economical extender for ram semen. These studies with reconstituted skim milk are preliminary in nature and no conclusions can be drawn until fertility tests can be made.

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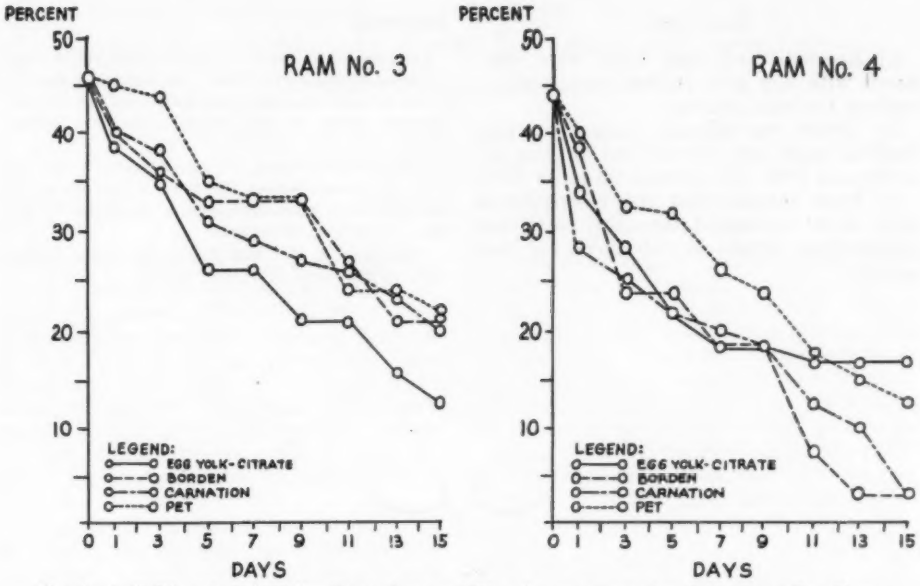
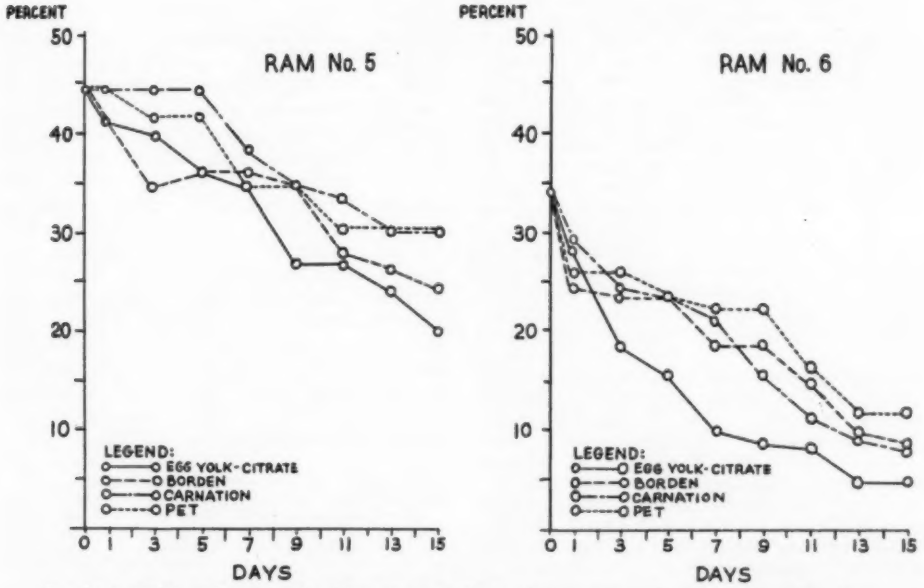
Supported in part by funds from the regional project S-29 entitled "Improving the Production of Early Milk Fat Lambs," a cooperative study by agricultural experiment stations in the southern region and the U.S.D.A.



Graphs 1 (left) and 2 (right)—Percentage motility of ram semen stored in egg yolk-sodium citrate, Borden, Carnation, and Pet skim milk extenders for a 15-day period at 38 F.



Graphs 3 (left) and 4 (right)—Percentage motility of ram semen stored in egg yolk-sodium citrate, Borden, Carnation, and Pet skim milk extenders for a 15-day period at 38 F.

SUMMARY

1) Reconstituted skim milks were compared with egg yolk-sodium citrate as extenders for ram semen.

2) Semen was collected from 4 yearling Suffolk rams and maintained in the extenders at 38 F. for periods up to 15 days.

3) Data showed that the reconstituted skim milks compared favorably with egg yolk-sodium citrate as extenders for ram semen.

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Distribution of Aqueous Penicillin and Penicillin in Oil in Normal Goat Udders Following Intramammary Injection—An Autoradiographic Study

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DURING THE TIME that intramammary antibiotic treatment of udders has been practiced, the problem of whether it is more suitable to use penicillin dissolved in water or suspended in oil has occasionally been discussed. Both types of treatment have attracted support. By using older techniques, however, it is difficult to estimate the distribution of penicillin within the udder when administered in either of these vehicles.

As it has been shown that penicillin in oil is generally excreted more slowly from the udder, the conclusion has been drawn that it is not necessary to give penicillin in oil as often as aqueous penicillin. Moreover, it is easy to manufacture directly useable preparations of penicillin in oil while aqueous penicillin solutions must be freshly prepared for each treatment because of the instability of penicillin in that form. These circumstances have contributed toward a greater use of penicillin in oil in practical therapeutics. The supposition has also been advanced that oil suspensions of penicillin carry penicillin up to the dorsal portions of the udder more easily because of their low specific weight, although no experimental foundation for this theory is available. By making microbiological determinations of the penicillin concentration in excised portions of frozen udders, Schipper [2] came to the opposite conclusion. In his experience, penicillin in oil penetrated incompletely up into the dorsal portions while aqueous penicillin was distributed more evenly throughout the udder.

The use of radioactively labeled penicillin and autoradiographic techniques is better than the use of biological methods for the careful localization of penicillin in relation to morphological features of tissue. The present report describes investigations carried out to study the distribution of penicillin labeled with the radioactive isotope S^{35} in normal goat udders using autoradiographic methods. It has seemed desirable to compare the penicillin content in various parts of the udder on a single autoradiographic survey film.

MATERIALS AND METHODS

Penicillin labeled with the radioactive isotope S^{35} was prepared. Both aqueous solutions and oil suspensions of the radioactive penicillin were introduced, through the teat canal, into actively lactating goat udders. At various intervals after injection, the animals were killed and the udders frozen by placing them in carbon dioxide-acetone. The udders were sliced in a freeze room and the slices, after drying at —10 C., were exposed by being pressed against photographic (x-ray) film.

Penicillin, labeled with S^{35} isotope, was prepared by biosynthesis in a half synthetic medium, the same method as that described earlier by one of the authors.[3] Carrier-free $Na_2S^{35}O_4$ (Harwell) was added to the medium and the level of stable sulfur in the medium kept low. Cultivation was carried out under conditions which favor the formation of benzyl penicillin.

Using a piperidine salt, the penicillin was precipitated out practically entirely as crystalline sodium salt. Paper chromatographic methods demonstrated the level of benzyl penicillin to be more than 90 per cent, with the remainder as hydroxybenzyl penicillin.

The specific activity of the radioactive penicillin obtained was 0.05 C/Gm. (Curie/gram) penicillin. The composition of the oil suspension was as follows: oleum arachidis, 5.0; aluminum monostearate, 250.0; tween 80, 5.8.

A dose of 10 mg. of penicillin, corresponding to a radioactive dose of 500 μC, was introduced into both udder halves of healthy goats. The same dose was used for both vehicles and the amount of vehicle was 3 ml. in every instance. The udders were not milked out before injection. One half of each udder was treated and, seven hours later,

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* The corresponding American titles would be D.V.M. and Ph.D.

† The corresponding American title would be D.V.M.

Fig. 1A—Autoradiograph showing distribution one hour (left) and eight hours (right) following the injection of S^{35} penicillin in 3 ml. of oil. There is good distribution throughout the entire udder, with higher concentration dorsally than ventrally, and in the milk ducts than in the parenchyma. The white areas are penicillin-filled; black areas, penicillin-free portions. x ½.

Fig. 1B—Corresponding tissue section. x 2/5.

the other. After an additional hour, the goats were killed and the entire udders were placed in a large container filled with acetone-carbon dioxide ice (—70 C.). Attention was directed toward maintaining the normal topography of the udders as far as possible. After being kept in the freeze mixture for sufficient time (about two hours) to ensure complete freezing, the udders were transferred to a cold room maintained at —10 C. The anterior surfaces of the udders were leveled off and the entire glands were placed on a large, metal object stage, especially constructed for a Leitz Grundschlitten microtome. The udder was fixed in place by slices of wet absorbent cotton placed around it on the object stage. The whole was then placed in the carbon dioxide snow and the procedure repeated until the lower part of the udder was encased in a supporting shell of ice.

A level surface was prepared on the upper surface of the frozen gland. Sections approximately 50 μ thick were prepared by sticking a broad tape (Scotch cellulose tape) onto the surface and then slicing under, and parallel to, the tape which served to hold the whole section together.[1,2] By this means, sections extending over both udder halves could be obtained from various levels of the glands. The distribution of penicillin in both halves and their various portions could thus be estimated on a single autoradiograph, although the penicillin had been introduced into each half at a different time before death.

The sections were dried at —10 C., the same temperature at which exposures were made. For exposure, the slices were pressed against no-screen x-ray film (Kodirex, Kodac, England) in metal presses for 14 days. After exposure, the film was

Fig. 2A—Autoradiograph showing distribution one hour (left) and eight hours (right) following the injection of S^{35} penicillin in 3 ml. of water. There is good distribution throughout the entire udder, with higher concentration ventrally than dorsally; uniform distribution in the milk ducts and parenchyma. x 2/5.

Fig. 2B—Corresponding tissue section. x ½.

separated from the sections, developed in Kodak D 19, and fixed. The sections were then transferred from tape to glass slides and stained.[1, 2] The autoradiographs are shown beside their corresponding stained sections (fig. 1 and 2.)

RESULTS AND DISCUSSION

Both the penicillin in oil (fig. 1) and the aqueous penicillin (fig. 2) showed a uniform distribution and reached all portions of the parenchyma. The interstitial tissues, blood vessels, and lymph nodes displayed no detectable penicillin. No significant difference in distribution between the autoradiographs made one and eight hours after injection could be seen for either the aqueous penicillin or the penicillin in oil.

A few differences could, however, be noticed between the distribution of penicillin introduced as an aqueous solution and that introduced at the same time as a suspension in oil. In the autoradiograph prepared from glands treated with the oil suspension, the branches of the milk ducts were especially accentuated. There was more penicillin in the milk ducts and less in the parenchyma following injection with penicillin in oil than with aqueous penicillin. In view of the large doses which are now used, the concentration of peni-

cillin suspended in oil is probably adequate within the parenchyma.

Penicillin in oil is more concentrated in the dorsal portions, presumably on account of the lower specific weight of the oil, while the aqueous penicillin is more concentrated in the ventral portions.

The distribution of penicillin within diseased udders will be dealt with in a following paper.

SUMMARY

Radioactive penicillin, labeled with isotope S^{35} and prepared both as an aqueous solution and as a suspension in oil, has been introduced through the teat canal into normal goat udders. The distribution of the penicillin, one and eight hours after injection, has been studied by autoradiographic methods. A fairly uniform distribution was obtained with both aqueous penicillin and penicillin in oil.

Penicillin in oil was concentrated more in the dorsal portions of the udders and was found in the milk ducts rather than in the parenchyma to a somewhat greater degree than was the case for penicillin in aqueous solution.

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Experimental Leptospirosis. III. Caprine *Leptospira Pomona* Infection

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CAPRINE LEPTOSPIROSIS is either a rarity or has not been detected in the United States. The disease, *per se,* will probably remain of minor economic importance since the goat population, in relation to that of cattle or swine, is small and goat milk is a specialty item in this country.

Leptospira pomona agglutinins have been found in the serums of apparently normal goats, according to van der Hoeden.[1] However, serological surveys of animals in the United States do not include data pertaining to goats. *Leptospira grippotyphosa* infection is responsible for unthriftiness, abortion, and death of goats in Israel.[2] The infection is prevalent, as indicated by serological surveys, and causes considerable economic loss.[2] Inappetence, fever, jaundice, and hemoglobinuria further characterize caprine *L. grippotyphosa* infections. Serum agglutinins may be observed at the 1:30,000 level, while whey agglutinins reach a level of 1:2,500. Approximately 59 per cent of 115 goats in one Israeli village were found to have serum antibody titers of 1:3,000 or higher.[2] Infections were experimentally established in 2 goats. Icterus, hemoglobinuria, and death occurred in 1 animal, while characteristic "greyish pinpoint areas" were observed on the surfaces of the kidneys of the other after 46 days.[2]

Goats also may be infected with *L. pomona* through contact with experimentally infected calves.[3] Therefore, goats should be considered as potential reservoirs of the leptospiroses for other host species. The following investigations were undertaken to ascertain the role of the goat in the transmission of leptospirosis (*L. pomona* infection) and to study the course of the disease in this species.

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MATERIALS AND METHODS

Five apparently normal goats served as experimental animals. Doe 988 was approximately 2 years old, bucks 820, 995, and 889 were 6 to 8 months of age, while buck 666 was 4 months old. The serums of these animals did not contain antibodies for either *L. pomona* or *Leptospira icterohaemorrhagiae* AB prior to initiation of the work.

Four animals (820, 889, 988, and 993) were exposed via the subcutaneous route. Each received 5.0 cc. of heparinized infected guinea pig blood. The inoculum represented the pooled blood from 4 guinea pigs exsanguinated during the febrile phase (105 to 106 F.) of the infection. *Leptospira pomona*, Wickard,[5] bovine origin, served as the infecting agent. When the inoculum was diluted 10^{-3} and injected intraperitoneally into 7, 6-week-old hamsters, 100 per cent infection resulted. A 10^{-4} dilution failed to infect any of 7 hamsters as determined by the agglutination-lysis test three weeks following inoculation.

Goat 666 was kept in a 10- by 13-foot, concrete-floored room with 3 *L. pomona*-infected hogs. After approximately 30 days of contact, the transmission had occurred as evidenced by the development of serum agglutinins in the goat.

Rectal temperatures were taken daily. Blood (0.02 to 0.1 cc.) was cultured in each of five tubes of Chang's fluid medium[4,5] on postinoculation days 7, 8, 10, and 13. The cultures were incubated at 30 C. and examined using dark field microscopy (x 600) at 14 days and again at 28 days to detect leptospiras. Urine, homogenized kidney, and pooled homogenized liver and spleen suspended in sterile 0.85 per cent sodium chloride solution were inoculated intraperitoneally into 5 to 7 guinea pigs or hamsters. Approximately 21 days following inoculation the animals were exsanguinated and their serums examined for the presence of *L. pomona* agglutinins. If antibody was demonstrable, the initial inoculum was considered to have contained *L. pomona.*

The tube agglutination-lysis test employing living antigen (*L. pomona*, strain Johnson[5]) was conducted at appropriate intervals on the serums of the goats. The tubes were incubated in a water bath at 37 C. for two hours and then examined. The entire contents of each tube (0.1 ml. of serum dilution with 0.1 ml. of antigen) were emptied onto a clean glass slide. The fluid was examined at x 100 using a microscope fitted with an Abbe condensor into which was inserted a star diaphragm to produce a modified dark field illumination. The leptospiras were agglutinated or lysed or showed both phenomena. Kidneys, livers,

spleens, brains, spinal cords, lymph nodes, and adrenal glands were fixed in Zenker's fluid, Carnoy's fluid, and isotonic, neutral formalin. Tissue sections were stained with hematoxylin and eosin, Sudan IV for fat, Bauer's glycogen stain, Verhoeff's elastic stain, Heidenhain's aniline blue for collagen, and by the parasilver-impregnation technique for spirochetes.

RESULTS

Characteristic clinical manifestations of leptospirosis, e.g., depression, anorexia, polypnea, hemoglobinuria, and icterus, were not evident. A mild diarrhea was observed in 1 goat (889) on postinoculation day 4. Rectal temperatures of goats 820, 889, and 995 were slightly elevated at some time during the six- to 10-day interval following inoculation. Goat 980 maintained a normal temperature during the entire course of the experiment. Goat 666 which was infected during exposure to 3 *L. pomona*-infected hogs showed no outward signs during the exposure period of ± 31 days (table 1).

Hematological findings determined on day 7 for the 4 inoculated animals were normal for erythrocytes, blood hemoglobin levels, total white blood cells, and differential white blood cell counts.

TABLE 1—Bacteriological Findings for Goats Experimentally Infected with Leptospira Pomona

Goat	Urine*	Kidneys*	Liver-spleen*
820	Pos.: 14 days	Pos.: 14 days	Neg.: 14 days
995	Pos.: 26 days	Pos.: 26 days	Neg.: 26 days
889	Pos.: 31 days	Pos.: 31 days	Neg.: 31 days
980	Neg.: 39 days	Neg.: 39 days	Neg.: 39 days
666†	Pos.: 9/6/56‡	Pos.: 9/6/56	Pos.: 9/6/56

* Guinea pig or hamster inoculation technique. † Transmission from infected hogs (contact infection occurred on ± Aug. 6, 1956). ‡ Leptospiras demonstrable by dark field microscopy in considerable numbers (x 600) in uncentrifuged sample.

Leptospiras were not isolated from the blood of the goats on postinoculation days 7, 8, 10, or 13. A mild febrile response (103.6 to 103.8 F.) was observed on day 8 in goats 820, 995, and 889. The temperature of 980 remained normal (101.0 to 102.0 F.).

The urine and kidneys of 820, 995, and 889 contained leptospiras at the time of necropsy. The liver-spleen emulsions of the 4 inoculated goats did not contain lep-

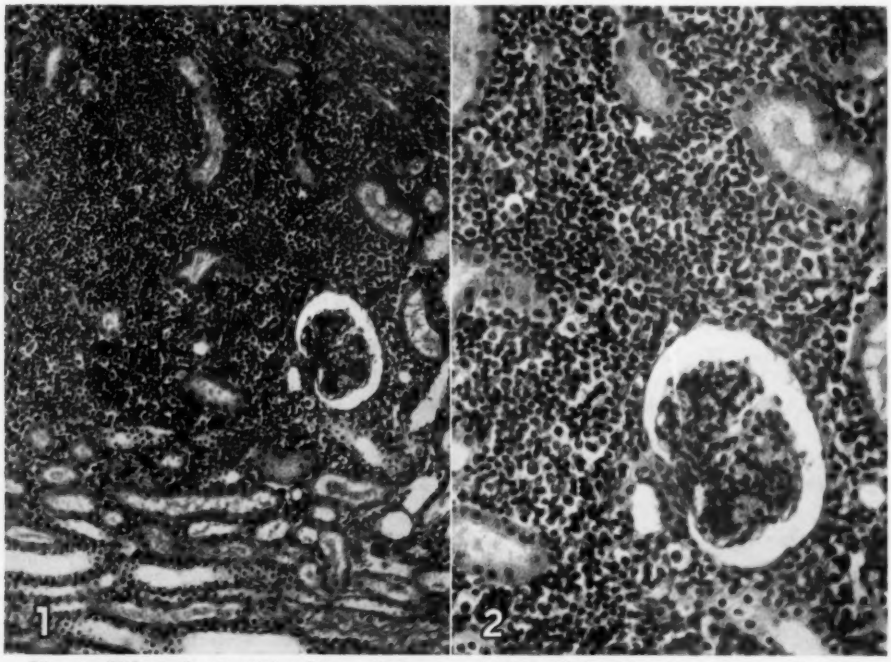

Fig. 1—Kidney (goat 889) with marked accumulations of lymphocytes, plasma cells, and mononuclear phagocytes. Note the atrophy and disappearance of tubules. x 120.

Fig. 2—Higher magnification of fig. 1. x 220.

tospiras as determined by animal inoculation. The tissues and urine of 988 were negative for leptospiras at 39 days (table 1).

Goat 666 was shedding large numbers of leptospiras in the urine approximately 31 days after contracting the disease from infected hogs. Dark field microscopic examination of uncentrifuged urine readily demonstrated considerable numbers of the microorganisms in each field at x 600. The kidneys and liver-spleen homogenate also contained leptospiras at necropsy (table 1).

Leptospiral antibodies were evident in the blood serums of goats 995 and 988 as early as day 7 and had reached at least the $1:10^{-4}$ level in all 4 animals by day 10. On day 13, all serum titers were $1:10^{5}$. On day 31, the agglutinin levels were descending in 2 animals (889 and 988) which had not been killed. At approximately 31 days following transmission, the serum-agglutinin level of goat 666 showed no evidence of diminishing, while urinary leptospiral agglutinins were present at 1:5 (table 2).

Gross pathological alterations, attributable to leptospirosis, were confined to the

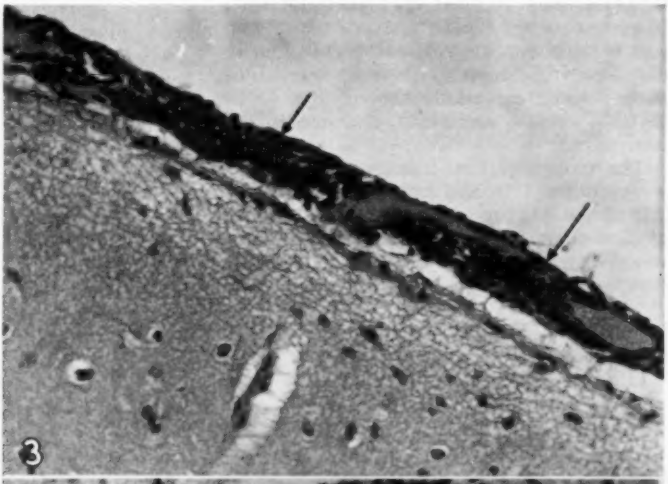

Fig. 3—Meninges (goat 820) revealing a marked accumulation of lymphocytes (arrows) and congestion. x 220.

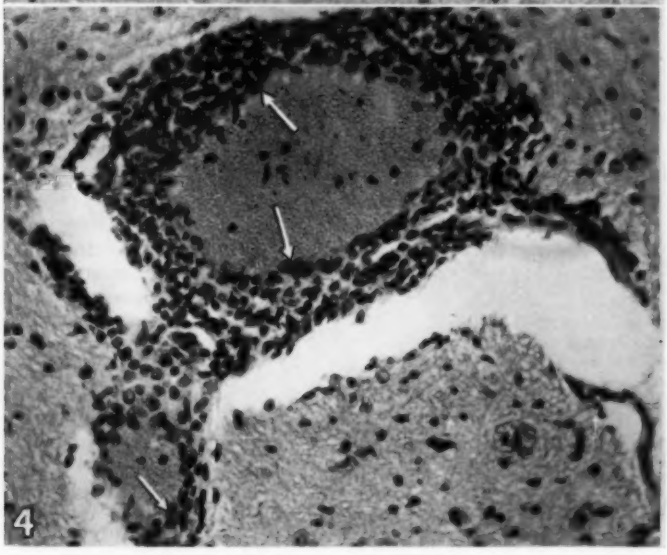

Fig. 4—Medulla oblongata (goat 820) showing a marked perivascular cuffing (arrows) with lymphocytes, a few macrophages, and plasma cells. x 220.

TABLE 2—Serum Agglutinin Titers for Goats Infected Experimentally with Leptospira Pomona

Goat	Postinoculation day								
	0	7	8	10	13	14	26	31	39
820	N*	N	1†	6	8	8	....	....	....
995	N	1	....	6	8	....	7	....	....
889	N	N	....	4	8	....	....	6	....
988	N	1	....	6	8	....	....	4	3
Goat	Date:		8/6	8/14	8/29	9/4	9/6		
666‡			N	2	4	4	6		

* N = negative at the 1:10 serum dilution. † Titer expressed as the negative exponent to the log base 10; agglutination or lysis of 25 per cent agglutination or lysis of antigen was considered positive. ‡ Goat infected during contact with *L. pomona*-infected hogs on ± Aug. 6, 1956.

kidneys. The cortical surfaces contained from one to six small, grayish white foci which measured approximately 1.0 mm. in diameter. On sagittal section, several of these lesions extended through the depth of the cortex and into the medullary portion.

The most significant microscopic lesions were confined to the kidneys and brains. All of the kidneys contained focal lesions of less than 2.0 mm. in diameter. These were characterized by accumulations of lymphocytes, plasma cells, and mononuclear phagocytes (fig. 1 and 2). Many segments of the nephrons in these areas were undergoing atrophy and necrosis. An increase of protein in the glomerular subcapsular spaces and lumens of the tubules was observed. A small number of the epithelial cells of the nephrons were pyknotic. In 2 goats, the arteries in the medulla showed a hyperplasia of the smooth muscle of the media. Hemorrhage into the tubular lumens was seen occasionally.

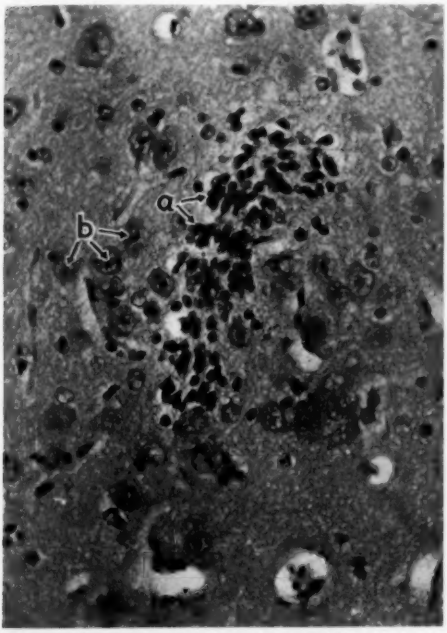

Fig. 5—Cerebrum (goat 995) exhibiting a proliferation of oligodendroglia cells (a) among numerous astrocytes (b). x 220.

Goats 889, 995, 820, and 886 showed microscopic lesions in the brain. In goats 889 and 820, the cerebrum, cerebellum, meninges, hippocampus, and medulla showed perivascular cuffing with lymphocytes, plasma cells, some mononuclear phagocytes, and edema (fig. 3 and 4). The nerve cells, however, did not show any recognizable changes. In goats 995 and 666, some perivascular hemorrhage had occurred in the medulla oblongata. An increase of the oligodendroglia cells was observed in 3 animals (fig. 5). Goat 820 had two lesions in the medulla oblongata which contained numerous gitter cells. Fatty metamorphosis of the hepatic cells at the center of the lobules occurred in goat 995. Small areas of fibrosis were observed in the adrenal glands of goats 995 and 889.

DISCUSSION

Goats can be experimentally infected with *L. pomona*. The disease also has been transmitted to goats by contact with calves [3] and swine. Although we are not aware of field cases of caprine *L. pomona* infection, the possibility of their occurrence can not be dismissed.

The clinical course of leptospirosis in goats tends to be inapparent. Gross pathological changes are minimal and are difficult to detect unless the kidneys are closely scrutinized.

Bacteriological examinations offer ample proof of the susceptibility of goats to *L. pomona* and demonstrate the presence of the agent in their tissues. Failure to demonstrate leptospiremia was due to either the exceedingly few leptospiras present in the blood or the presence of blood antibody at the times when cultures were made. Approximately 0.1 cc. of blood was inoculated into each of five tubes of medium. This quantity might not have contained sufficient microorganisms to enable growth. Serum antibodies were evident for 3 goats on the first day of blood culture, and their

presence may have either destroyed leptospiras in the blood or inhibited growth in the medium.

Serological examinations are the most practical means of detecting the disease. The rapid development of serum leptospiral agglutinins and evidence of ascending titers is comparable to that observed for cattle, swine, and sheep.

The gross and microscopic characteristics of experimental *L. pomona* infections in goats have not been previously described. Van der Hoeden in Israel [2] reported that in caprine *L. grippotyphosa* infections the macroscopic alterations were more extensive than those produced by *L. pomona*. These changes included emaciation, intense icterus, congestion, edema of lymph nodes, and an interstitial nephritis with hemorrhages. Microscopically, the lesions in the kidneys were more extensive than those produced by *L. pomona*. The livers reportedly showed congestion, degeneration, and necrobiosis at the center of lobules. The severity of the lesions may have been accentuated by a verminous bronchopneumonia.

The small, grayish white, circumscribed areas in the kidneys of goats are similar to those seen in cattle and sheep.[11-16] The microscopic lesions are similar to those observed in sheep but are not so extensive.[16]

Clinically, recognizable central nervous system disturbances were not observed in the goats, yet mild to severe lesions were detected microscopically. Signs of meningitis have been reported in man,[12, 13, 15] cattle,[14] and swine [8] infected with leptospiras. It is possible that, if more brains from leptospirosis cases were examined histologically, more cases of encephalitis would be found. Unfortunately, the brains were not cultured to eliminate other infections. However, based upon previous reports in the literature and two unreported experimental leptospirosis infections in sheep,[16] it would appear that this disease can cause changes in the central nervous system.

Workers in Australia,[7] Israel,[2] and the United States [9] have described extensive necrosis of the liver in sheep, goats, and cattle, respectively, but such damage was absent in these experimental goats. Leptospiras were found in the liver and spleen of goat 666, but lesions were absent.

The duration of the carrier state for goats is unknown. However, the number of leptospiras contained in the urine of 1 goat (666) at 31 days was equal to that observed in the urine of infected swine. At this time, this goat represented a potential equal to the hog in ability to disseminate the disease to other animals.

SUMMARY

The course of *Leptospira pomona* infection in goats appears to be occult. Gross pathological changes, which were minimal and were observed only in the kidneys, appeared as scattered, grayish white foci which, on section, extended through the depth of the cortex and occasionally into the medulla and consisted of foci of lymphocytes, plasma cells, and a few macrophages. The brains of 4 goats showed meningitic and encephalitic lesions. The urine may contain tremendous numbers of leptospiras for at least 30 days following infection. The kidneys and, in some instances, the liver or spleen may contain leptospiras.

Transmission from infected swine to a goat has been established. Therefore, the goat represents a potential source of *L. pomona* infection for susceptible hosts. In areas where the goat population is high, this species should be considered as a reservoir of *L. pomona* infection.

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Prevalence of Agglutinins in Canine Serums to Serotypes Other than *Leptospira Canicola* and *Leptospira Icterohaemorrhagiae*—Report of Isolation of *Leptospira Pomona* from a Dog

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ALTHOUGH INFECTIONS with *Leptospira canicola* and *Leptospira icterohaemorrhagiae* have been observed extensively in dogs, the presence of other leptospiral serotypes has rarely been observed in canine populations of the world outside of southeastern Asia.[2,3] In this country, previous surveys have indicated that leptospiral infections in dogs are due predominantly to *L. canicola* and, less frequently, to *L. icterohaemorrhagiae*.[3]

In a recent report, Alexander *et al.*[3] presented presumptive evidence of canine infections with serotypes other than *L. canicola* and *L. icterohaemorrhagiae*. As reported by these authors, the failure to detect additional antibodies against other "murine" and "bovine" serotypes reflects, in large measure, the limited specificity of agglutination procedures in which the testing antigens were restricted to the sole employment of *L. canicola* and *L. icterohaemorrhagiae*.

In view of the above findings, a serological study was conducted on a group of "normal" dogs from Pennsylvania to determine the distribution of antibodies against diverse serotypes and to ascertain the presence of serotypes other than *L. canicola* and *L. icterohaemorrhagiae* in dogs. The serological test results are presented. In addition, this paper reports the recovery of *Leptospira pomona* from a naturally infected dog.

MATERIALS AND METHODS

Source of Dogs.—A total of 357 normal dogs of mixed breed, routinely obtained for research purposes by the Walter Reed Army Institute of Research, were studied. These dogs, approximately 1 to 3 years old, were obtained during the period from March to September 1956, inclusive, most of them from rural areas in Pennsylvania but a few from similar areas in neighboring states. Detailed histories on each animal were not available prior to the time the dogs were delivered, other than that immunizations against rabies, canine distemper, and canine hepatitis were given prior to shipment. No clinical signs referable to acute leptospirosis were observed in any of the animals tested.

Serological Survey.—Serums were examined for the presence of leptospiral agglutinins by the microscopic-agglutination (agglutination-lysis) test employing live leptospiral organisms. The techniques of the test are described in detail elsewhere.[13] Serums were diluted fourfold to provide final serum dilutions ranging from 1:25 to 1:6,400 after addition of the antigen. Serums positive at 1:6,400 were retested at higher dilutions until an end point was reached. Agglutination reactions at 1:100 or greater were considered significant.

All of the serums (357) were examined for agglutinins against seven leptospiral antigens. These were *L. pomona*, S-91; *L. autumnalis*, Fort Bragg; *L. ballum*, HS-36; *L. canicola*, Hond Utrecht; *L. icterohaemorrhagiae AB*, Wijnberg; *L. grippotyphosa*, Moscow V; and *L. sejroe*, Mallersdorf. In addition, 63 were tested against nine other leptospiral antigens: *L. semaranga*, R-S 173; *L. celledoni*, Celledoni; *L. bataviae*, Van Tienen; *L. djasiman*, Djasiman; *L. sentot*, Sentot; *L. pyrogenes*, Salinem; *L. alexi*, HS-616; *L. borincana*, HS-622; and *L. javanica*, Bataviae 46. Serological tests were also performed on paired samples of serums obtained at various intervals from a limited number of serologically positive animals. Only the initial serums were included in the statistical data.

Isolation Procedures.—Attempts were made to recover leptospiras from the urine of 31 dogs that were serological reactors to serotypes other than *L. canicola* and *L. icterohaemorrhagiae*. For this purpose, urine from each dog was obtained by means of a sterile catheter. The samples were examined by darkground microscopy for the presence of leptospiras, and 0.1- to 0.2-ml. amounts were inoculated intraperitoneally into each of 3, 15- to 20-Gm. hamsters *(Cricetus auratus)*. Blood samples were obtained from the heart of each hamster on postinoculation days 4, 7, 11, and 28 and cultured on three tubes of Fletcher's semisolid medium.[9] In addition, hamster kidney sections were cultured on day 28. Cultures were incubated and examined by conventional methods.[12] The above procedures were repeated on replicate

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urine samples obtained from a limited number of animals at three- to seven-day intervals. A total of 57 urine samples was examined. Of the 31 animals, 9 were were subsequently killed, at which time aqueous humor, kidney tissues, and urine, removed aseptically from the bladders, were inoculated into Fletcher's medium. Attempts were also made to recover leptospiras from suspensions of kidney tissues via hamster inoculation procedures.

Identification of Isolate.—The antigenic characteristics of a leptospiral isolate were determined on the basis of results of cross-agglutination and agglutinin-absorption techniques. The preparation of antiserum in rabbits, cross-agglutination, and agglutinin-absorption tests were conducted according to prescribed techniques employed in this laboratory.[1]

RESULTS

Serological Survey.—Of the 357 canine serums tested for the presence of leptospiral antibodies, 58 (16.2%) showed significant titers of 1:100 or greater. The distribution of the predominant titers in seropositive dogs, among the screening antigens, is shown (table 1). In this sampling of Pennsylvania dogs, the higher percentage of *L. autumnalis* than the combined percentages of *L. canicola* and *L. icterohaemorrhagiae* reactors is indeed striking and, to the authors' knowledge, has not been observed in previous serological surveys in dogs, in this hemisphere. Predominant *L. autumnalis* agglutinins were distributed in approximately 41 per cent of the seropositive animals in contrast to the 24 per cent and 12 per cent that were positive against *L. canicola* and *L. icterohaemorrhagiae,* respectively. Lesser percentages of positive dogs showed predominant agglutinins against *L. grippotyphosa* (8%) and *L. ballum* (3%) and 10 per cent of the dogs showed agglutinins of the same order against multiple serotypes.

Cross-agglutinins against the screening antigens were frequently found. These were particularly evident among higher titer positive serums. Serums showing maximum titers against either *L. autumnalis* or *L. grippotyphosa* frequently cross-reacted against the other respective strain as well as against *L. pomona.* Typical patterns of cross-reactivity of *L. autumnalis* and *L. grippotyphosa* seropositive serums are shown (table 2). Predominant *L. canicola* reactors, in many instances, showed a lesser titer against *L. ballum* or *L. icterohaemorrhagiae* or both. Similarly, serums showing highest titers against *L. icterohaemorrhagiae* reacted to lower titers against one or more of the following antigens: *L. canicola, L. ballum,* and *L. autumnalis.* Extending the number of testing antigens in a sampling of 63 canine serums elicited additional lower order cross-reactions against *L. djasiman* or *L. sentot,* or both, in some of the serums reacting primarily against *L. autumnalis.* Similarly, serums positive against *L. icterohaemorrhagiae* cross-reacted with one or more of the following antigens: *L. sentot, L. pyrogenes,* and *L. javanica.* In addition, serums manifesting highest titers against *L. canicola* showed coagglutinins against *L. pyrogenes.*

The employment of multiple screening antigens revealed a significantly higher prevalence of serological reactors than would ordinarily be demonstrated by the sole use of *L. canicola* and *L. icterohaemorrhagiae* as testing antigens. The number and percentage of seropositive reactors, of the total number of dogs examined, that could be detected by use of each of respective screening antigens are shown (table 3). Thus, the restrictive employment of *L. canicola* and *L. icterohaemorrhagiae* antigens would have disclosed a prevalence ratio of agglutinins of 8.4; approximately half of the actual percentage (16.2) of positive reactors. The employment of the aforementioned nine antigens, in addition to the seven screening antigens, did not affect the percentage of seropositive reactors in a sampling of 63 dogs. Positive reactions exhibited against these additional antigens were also elicited by the screening antigens.

Identification of Isolate.—Leptospiras were isolated from 1 dog (3341) by direct culture of bladder urine obtained at necropsy. Attempts to isolate leptospiras from the other 30 dogs were unsuccessful. No

TABLE 1—Prevalence of Leptospiral Agglutinins in 357 Canine Serums

No. of serums showing	Leptospiral serotypes								Positive	
	L. pomona	*L. autumnalis*	*L. ballum*	*L. canicola*	*L. icterohaemorrhagiae*	*L. grippotyphosa*	*L. aejroe*	Other	(No.)	(%)
Predominant agglutinin titers	0	24	2	14	7	5	0	6*	58	16.2

* Reacting equally to *L. pomona* and *L. autumnalis* (1); *L. ballum* and *L. icterohaemorrhagiae* (1); *L. autumnalis* and *L. grippotyphosa* (2); *L. autumnalis* and *L. canicola* (1); and *L. ballum* and *L. canicola* (1).

TABLE 2—Cross-Agglutination Reactions in Paired Serums from 6 Canine Reactors Against L. Autumnalis, L. Grippotyphosa, or L. Pomona

Dog (No.)	Serum collected	Reciprocal of agglutinin titer against antigen		
		L. pomona, S-91	*L. autumnalis*, Fort Bragg	*L. grippotyphosa*, Moscow V
3341	4-19-56	—*	400	100
	5-15-56	100	400	100
1667	9-25-56	100	400	100
	10-26-56	100	400	100
3327	4-19-56	25	1,600	100
	4-30-56	100	400	100
3316	4-19-56	100	1,600	100
	4-30-56	400	6,400	400
1885	7-12-56	—	400	400
	8-20-56	25	400	400
1888	7-27-56	400	1,600	1,600
	8-20-56	100	1,600	400

* Negative (— = <100).

leptospiras were observed in the direct microscopic examination of urine samples. Paired serum samples, obtained 26 days apart from dog 3341, reacted to highest titer against *L. autumnalis* and showed lower order cross-agglutinins against *L. grippotyphosa* and *L. pomona* (table 2). The initial serum sample showed no detectable agglutinins against *L. pomona* at a dilution of 1:25, within the limitations of the methods employed.

The isolated strain (3341) employed as antigen was initially tested against 18 serotype rabbit antiserums routinely employed in this laboratory.[13] Based on the observed cross-reactions, strain 3341 was screened against ten additional serotype rabbit antiserums to elicit further relationships. The cross-agglutination reactions of strain 3341 (table 4) were indistinguishable from those of *L. pomona*, strain V-42. Similarly, antiserum prepared in a rabbit against strain 3341 displayed the typical agglutinin reactions (table 4) incited by *L. pomona* antigens in this host.[26]

For the definitive identification of the canine isolate, cross-agglutinin absorption studies were conducted with various serotype members of the pomona serogroup.[2] The results of agglutinin-absorption studies (table 5) indicate that strain 3341 is serologically homologous to the type strain *L. pomona*, Pomona, originally described by Clayton and Derrick.[6] The antigenic interrelationships of strain 3341 with other serotypes in the pomona group are consistent with previous observations on antigenic differences among strains of *L. pomona*.[2]

In view of the unequivocal identity of strain 3341 as *L. pomona*, the paucity of *L. pomona* antibodies and the presence of maximum titers against *L. autumnalis* in the original host serums were perplexing. Therefore, the two serum samples from dog 3341, as well as serums from 25 other dogs showing maximum titers against *L. autumnalis* or *L. grippotyphosa*, were retested against strain 3341 and the following four other antigenically dissimilar type strains within the pomona group: *L. pomona*, S-91; *L. pomona*, Pomona; *L. pomona*, C-B; *L. pomona*, C-164.[2] The agglutinin titers observed in serums with this array of *L. pomona* antigens were identical to those originally observed against *L. pomona*, S-91, employed as a screening antigen.

Further studies, employing agglutinin-absorption tests and animal-inoculation procedures provided additional evidence that the "heterologous" agglutinins in serums from dog 3341, as well as in other dogs, were provoked by infections with *L. pomona*. Absorption of serums from dog 3341, and 3 additional dogs showing a similar pattern of cross-agglutinins with antigen prepared from strain 3341, effected

TABLE 3—Distribution of Leptospiral Agglutinins Against Seven Serotype Antigens in 58 Serologically Positive Canine Serums*

Antigen	Positive serums	
	No.	Per cent of 357 serums
L. pomona	8	2.2
L. autumnalis	34	9.5
L. ballum	13	3.6
L. canicola	20	5.6
L. icterohaemorrhagiae	10	2.8
L. grippotyphosa	17	4.7
L. sejroe	0	0.0

* Includes predominant as well as cross-reactions at titer levels of 1:100 or greater.

TABLE 4—Cross-Agglutination Results on Leptospira Strain 3341

Rabbit antiserum or antigen from strain	Reciprocal of titer: Antigen * *L. pomona*, 3341	Antigen * *L. pomona*, V-42	Rabbit antiserum 3341 †
3341	25,600	25,600	25,600
L. pomona, V-42 ‡	102,400	25,600	25,600
L. pomona, Pomona ‡	25,600	25,600	25,600
L. pomona, C-B ‡	25,600	25,600	25,600
L. pomona, 285 ‡	25,600	6,400	25,600
L. pomona, S-91 ‡	25,600	6,400	25,600
L. pomona, C-164 ‡	25,600	25,600	25,600
L. autumnalis, Fort Bragg	100	400	1,600
L. autumnalis AB, Aki. A	400	400	400
L. autumnalis A, Rachmat	6,400	1,600	400
L. bangkinang	—	—	1,600
L. mooris §	—	Not tested	1,600
L. grippotyphosa, Moscow V	—	—	100
L. sentot	100	100	400

* Also negative (− = <100) against the following type rabbit antiserums: *L. andaman, L. semaranga, L. djatzi, L. hyos, L. djasiman, L. australas A, L. ballum, L. canicola, L. icterohaemorrhagiae AB, L. australis B, L. alexi, L. medanensis, L. javanica,* and *L. pyrogenes.*

† Also negative against the following antigens: *L. andaman, L. semaranga, L. celledoni, L. bataviae, L. djasiman, L. ballum, L. canicola, L. icterohaemorrhagiae AB, L. pyrogenes, L. alexi, L. borincana, L. sejroe,* and *L. javanica.*

‡ Antigenic characteristics of these strains are described elsewhere (Alexander *et al.*, 1957[2]).

§ New serotype in the *autumnalis* group isolated in Malaya (Alexander *et al.*, in press [4]).

the complete removal of the agglutinins against homologous and heterologous antigens. Finally, 2 normal dogs, approximately 5 years old, were experimentally infected with strain 3341 by the intraperitoneal inoculation with 10 ml. of a viable culture in Fletcher's medium. The agglutinin responses in these infected dogs, observed over a period of 17 weeks, were analogous to those seen in dog 3341 and in other Pennsylvania dogs.

Coagglutinin titers against *L. autumnalis* that were equal to or, more frequently, higher than those against the homologous strain are shown (table 6). Titers against *L. grippotyphosa* were lower than *L. autumnalis* titers but in some serums were equal to the *L. pomona* titers. Predominant titers against *L. autumnalis* persisted during the observation period of approximately four months. Agglutinins against *L. icterohaemorrhagiae* antigen were detectable in the serum of the 2 experimentally infected dogs at a titer of 1:100 for a period of four weeks in 1 dog and 15 weeks in the second. During this period, these titers were of the same order of magnitude as those against the other strains tested as shown (table 6). No clinical signs referable to acute leptospirosis were observed in the experimentally infected dogs.

DISCUSSION

The demonstration of *L. pomona* infection in a dog directs attention to the potential role of the dog in the infectious cycle of human and animal leptospirosis in this country. In addition to serving as an incidental host to *L. pomona,* a serotype that is primarily associated with livestock, dogs may, conversely, be responsible for cross-infections in larger domestic animals with the classic canine serotype, *L. canicola. L. canicola* infections in livestock, subsequently transmitted to human beings and traced to canine reservoirs of infection, have been observed in this country [7,25] and in Israel.[20-22] In addition, dogs may possibly serve as the intermediate link in the chain of transmission of leptospiral infections in larger domestic animals and man with serotypes that primarily parasitize various murine and other rodent hosts.

Leptospira pomona agglutinins in dogs have been observed in the United States [3,17] as well as in other parts of the world [5,8,10,18] and this serotype has been previously isolated from a dog in Indonesia.[15] In addition to *L. pomona, L. icterohaemorrhagiae,* and *L. canicola,* dogs can be infected, and can serve as potential sources of infection, with numerous other serotypes. These include *L. saxkoebing,*[14] *L. australis A,*[15] *L. bataviae,*[15] *L. autumnalis,*[9,28] *L. hebdomadis,*[27,28] *L. medanensis,*[27] and *L. sejroe.*[19]

In the formulation of measures for control and prophylaxis of leptospirosis in man, livestock, and dogs, the potential cross-infection hazards between species should be recognized. Effective control measures are contingent upon a better understanding of the prevalence of various serotypes in potential hosts. For this purpose, the sole employment of *L. canicola* and *L. icterohaemorrhagiae* antigens for the laboratory diagnosis and for serological surveys of canine leptospirosis and, similarly, the testing of livestock serums solely against *L. pomona* antigen, may not detect the presence of agglutinins provoked by antigenically dissimilar serotypes in the respective hosts. To ensure detection of agglutinins, it is important to employ multiple screening antigens which encompass a broad spectrum of cross-reactions. In this survey, the use of the additional

TABLE 5—Cross Agglutinin-Absorption Studies on Canine Isolate 3341 and Members* of the Pomona Serogroup

Antiserum		Reciprocal of titer against antigen					
Against	Absorbed with	3341	C-B	Pomona	V-42	S-91	C-164
3341	Unabsorbed	25,600	25,600	25,600	102,400	102,400	25,600
	3341	—†	—	—	—	—	—
	C-B	—	—				
	Pomona	100‡		100‡			
	V-42	—			—		
	S-91	400				—	
	C-164	—					—
L. pomona, C-B	Unabsorbed	25,600	25,600				
	3341	—	6,400				
	C-B	—	—				
L. pomona, Pomona	Unabsorbed	25,600		25,600			
	3341	—		—			
	Pomona	—		—			
L. pomona, V-42	Unabsorbed	102,400			102,400		
	3341	—			—		
	V-42	100‡			—		
L. pomona, S-91	Unabsorbed	102,400				102,400	
	3341	—				6,400	
	S-91	—				—	
L. pomona, C-164	Unabsorbed	25,600					25,600
	3341	—					400
	C-164	—					—

* Serological characteristics of *L. pomona* strains are by Alexander *et al.*, 1957.[3] † Negative (— = 100).
‡ Incompletely absorbed.

antigens—*L. pomona, L. autumnalis,* and *L. grippotyphosa*—uncovered more than half of the seropositive reactors. This laboratory now routinely employs the following six antigens in testing serums from livestock and dogs: *L. canicola, L. icterohaemorrhagiae, L. pomona, L. grippotyphosa, L. sejroe,* and *L. autumnalis.* Canine serums are also screened against *L. ballum.* Frequently, particularly during the conduct of serological surveys, samples will be spot-checked against the complete battery of 17 screening antigens employed for diagnosis in human leptospirosis.[13]

The results of this study focus attention on the limitations of the serological response of the host for identifying the infecting serotype. Thus, *L. pomona* was isolated from a dog that showed predominant agglutinins against *L. autumnalis* and lower titer cross-reactions against *L. grippotyphosa.* Cross-reactions against both of these serotypes, as well as against *L. icterohaemorrhagiae,* have been observed in serums from various hosts infected with *L. pomona.*[16, 24] Heterologous titers exceeding those of the homologous strain have been frequently observed. However, these so-called "paraspecific responses" are generally manifested the first six weeks after onset of disease; thereafter, homologous titers predominate.[11, 24] The persistence of predominant titers against heterologous strains up to 17 weeks, in 2 dogs experimentally infected with the *L. pomona* isolate, was unexpected and requires further elucidation.

On the basis of the serological findings in dog 3341, as well as the results of agglutinin-absorption tests on serums from 3 additional dogs, it is reasonable to assume that most of the dogs showing patterns of cross-reactivity against *L. autumnalis, L. pomona,* and *L. grippotyphosa* were infected with *L. pomona.* The remarkably higher prevalence of "*L. pomona*" than *L. canicola* reactors in this group of dogs may well reflect the rural origin of these animals. A rural area of enzootic *L. pomona* leptospirosis may provide greater opportunity for the spread of *L. pomona* infections in dogs. In this study, it was not possible to survey serums from other mammalian hosts in the same area. It is possible that results obtained reflect local enzootic conditions. Further studies on dogs located in rural areas of other parts of the country would contribute significantly to basic information on the epizootiology of leptospiral infections in animals.

It is of interest that leptospiruria was disclosed in only 1 of 31 dogs showing agglutinins against either *L. grippotyphosa, L. autumnalis,* or *L. pomona.* In contrast, a similar study conducted previously in this laboratory showed that approximately 11 per cent of the *L. cani-*

TABLE 6—Agglutinin Response in 2 Dogs Experimentally Infected with Strain 3341

Dog (No.)	Serum sample (weeks post-inoculation)	Reciprocal of agglutinin titer against antigen *					
		3341	*L. pomona* V-42	*L. pomona* S-91	*L. autumn.* Fort Bragg	*L. grippo.* Moscow V	*L. ictero AB* Wijnberg
1629	pre-inoc.	—	—	—	—	—	—
	1	1,600	1,600	1,600	1,600	400	400
	2	400	400	400	1,600	100	400
	3	400	400	400	1,600	400	400
	4	400	400	400	1,600	400	400
	13	400	400	400	1,600	100	100
	15	100	100	100	400	25	100
	17	400	400	400	400	100	25
1631	pre-inoc.	—	—	—	—	—	—
	1	400	400	400	400	100	400
	2	400	400	400	1,600	100	400
	3†	400	400	400	400	100	400
	4†	100	400	400	400	100	100
	13†	100	100	100	400	—	25
	15†	—	100	—	400	—	25
	17†	100	100	100	400	—	25

* No reaction at 1:25 (— = neg.) against *L. sejroe* and *L. ballum*. † Reaction at 1:25 against *L. canicola*.

cola seropositive reactors were infective.[3] These disparate findings may possibly reflect differences in the parasitic adaptation of the respective serotypes. This and other problems in the pathogenesis of *L. pomona* infections in dogs, posed by the findings of this study, warrant additional experimental and epizootiological observations.

SUMMARY

Leptospiral agglutinins were present in 16.2 per cent of 357 canine serums when tested against a battery of seven live leptospiral antigens. Predominant *Leptospira autumnalis* agglutinins were present in 41 per cent of the seropositive animals, in contrast to the 24 per cent and 12 per cent that were positive against *Leptospira canicola* and *Leptospira icterohaemorrhagiae*, respectively. Predominant agglutinin titers against *Leptospira grippotyphosa* (8%) and *Leptospira ballum* (3%) were also found.

A leptospiral isolate was obtained from the urine of 1 dog which had a predominant *Leptospira autumnalis* titer. This isolate was characterized as a strain of *Leptospira pomona* by cross-agglutination and agglutinin-absorption procedures.

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Cytopathology and Rate of Release of Infectious Canine Hepatitis Virus Grown in Dog Kidney Cells

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IT HAS BEEN REPORTED by Cabasso *et al.*,[1] Fieldsteel *et al.*,[5] and Muller and Thordal-Christensen,[7] that the virus of infectious canine hepatitis (ICH) multiplies readily in tissue culture. The growth of the virus is accompanied by specific cytological degeneration which can be neutralized by immune serum. Extensive immunological studies of ICH virus in tissue culture have been carried out by Fieldsteel.[4] As pointed out by Rubarth,[9] animals infected with this virus show specific cellular changes, consisting of intranuclear inclusions in the epithelial cells of the liver, and in the endothelial and reticuloendothelial cells in other organs. Thordal-Christensen [10] described cytopathic changes in plasma clot preparations of infected dog testicular cells, but extensive studies of cytopathology in tissue culture preparations have not been reported.

In this study, cells were observed microscopically, at specific time intervals after inoculation, to determine the lesions caused by virus. Methods similar to those used by Dunnebacke [3] with polio virus were used. Cultures of canine kidney cells were infected with a tissue culture-adapted strain of ICH virus. At periodic intervals, infected cover slips were removed for cytological studies, and tissue culture fluids were saved for virus assay. In this manner, the progress of cellular lesions was compared with the rate of release of virus.

MATERIALS AND METHODS

Cultures of canine kidney cells, prepared by the method of Dulbecco and Vogt,[2] were grown in flasks. The fluid in the flasks was changed four days later, and at the end of six days the cultures were removed from the flasks, using 0.25 per cent trypsin, and reseeded into Petri dishes containing cover slips.

Virus.—Infectious canine hepatitis (ICH) virus at the fifteenth tissue culture passage level was used in all experiments.* All mediums and buffer solutions contained 100 units of penicillin and 100 μg. of streptomycin per milliliter.

The cells were grown in Petri dishes (50 mm. in diameter), each containing three 18-mm. square cover slips covered by 4 ml. of medium. They were incubated at 37 C. in an atmosphere of 5 per cent CO_2. When these cultures attained confluent growth, after about three days, slips were transferred to individual Petri dishes. Cell counts were not made on these slips; however, each was examined to make certain that there was a continuous cell sheet over the entire slip. After placing the slips in their individual Petri dishes, each was washed with three changes of 37-C. phosphate buffer solution at 20-minute intervals to remove all serum. These Petri dishes were then divided into two groups. One half of the slips, designated "control group," were covered by 4 ml. of medium 199.[6] The other half were inoculated with 4 ml. of virus stock solution, which consisted of a 1:8 dilution in medium 199 of infected tissue culture supernatant fluid, containing approximately 10^6 tissue culture infective doses ($t.c.i.d._{50}$) before dilution. Infected and noninfected plates were placed in the incubator.

At the end of a two-hour virus-fixation period, the supernatant fluid was removed and stored at —20 C. Each slip was washed three times at 15-minute intervals with 4 ml. of warm phosphate buffer solution. The last wash of phosphate buffer was saved from each Petri dish for future assay of virus content. No serum was added to the cultures. Slips from one infected and one control culture were removed at timed intervals following infection. Slips, at this time, were placed in Bouin's fluid to fix the cells, and the medium from these cultures was saved for assay. One infected and one control slip were prepared simultaneously at each period. The slips were stained with iron hematoxylin and eosin for cytological study.

Virus titers were measured in all assays by making tenfold dilutions of material in medium 199, after which two to four tubes of canine kidney cells were inoculated with each dilution. Fifty per cent tissue culture infective doses ($t.c.i.d._{50}$) were calculated by the method of Reed and Muench.[8]

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* Virus obtained from Dr. A. H. Fieldsteel.

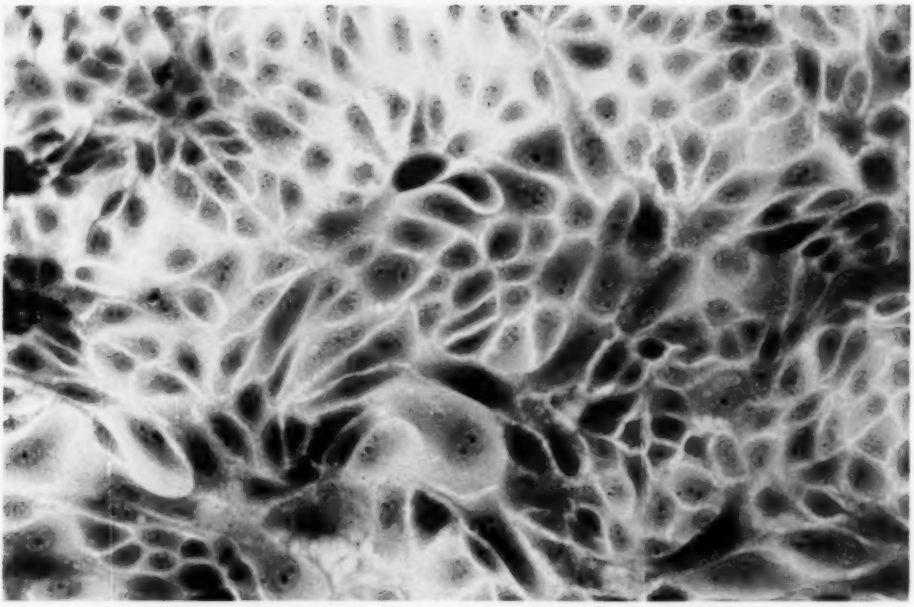


Fig. 1—Normal canine kidney cells grown on coverglass. x 375.

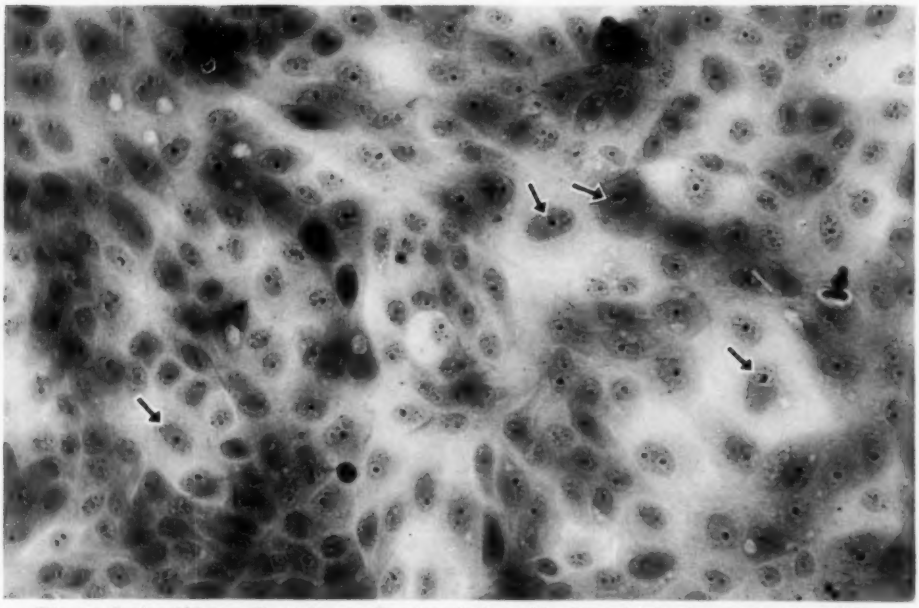
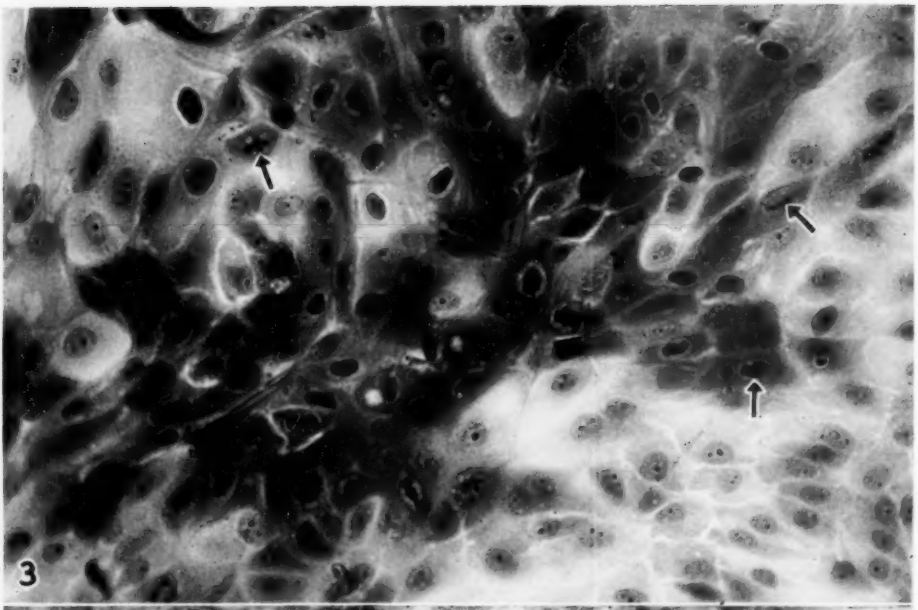


Fig. 2—Canine kidney cells 13 hours after inoculation with virus of infectious canine hepatitis. Notice swollen nuclei (arrows) and foamy appearance of chromatin. x 375.



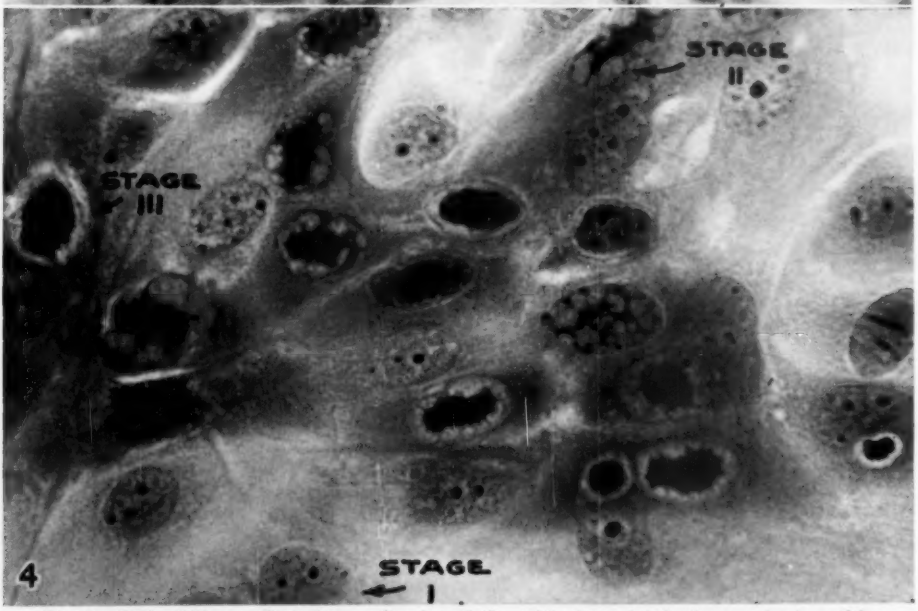


Fig. 3—Canine kidney cells 23 hours after inoculation with virus of infectious canine hepatitis. Notice the many large, dark-staining, intranuclear inclusions (arrows). x 375.

Fig. 4—Higher magnification of figure 3 to show nuclear detail of cells 23 hours after inoculation. Notice stages of nuclear response. x 950.

RESULTS

Cytology.—The stained cover slip preparations of the control and the infected cells were examined under the microscope to study the specific lesions caused by ICH virus. Inasmuch as many cells in the infected cultures dropped off the slides when lesions began to develop, total cell counts of entire slides were not attempted.

Control Cultures.—Control cultures, which exhibited none of the changes seen in the infected cells, consisted of sheets of cells with round to oval nuclei forming a mosaic pattern of flat cells, interspersed with whorls of spindle-shaped cells which, in many areas, appeared to be layered on top of each other (fig. 1). These were considered rests, where the original clumps of cells settled down on the glass to form the colonies from which new cells developed. Each nucleus contained one to four dark-staining nucleoli. The chromatin formed a fine lacy network of uniform density. The nuclear membrane was thin but definite. The area covered by cytoplasm was variable, depending on the density of cell growth. Where cell growth was sparse, the area of cytoplasm was about four to five times that of the nucleus. In areas of dense growth it was smaller. The borders of the cytoplasm were clearly defined and usually angular, with the circumference of the cells arranged in triangular, rectangular, or polyhedral form. There was a narrow, definite, unstained margin between most cells, and between many of the cells, narrow tendrils of cytoplasm, resembling intercellular bridges, were visible. Some cells contained clear, smooth-walled lipoid vacuoles in the cytoplasm. There was a definite variation in cell shape from round to polyhedral to spindle. However, transitional forms could be seen between these extremes. It was noticed that, wherever cell growth was less dense, all cells were epithelial in appearance while, in areas of dense growth, most cells were elongated and fibroblastic in shape.

Infected Cultures.—No specific cellular changes were observed 13 hours after infection. There did appear, however, to be some enlargement and irregularity of nucleoli, accompanied by a change in appearance of the chromatin network. This material in some cells appeared to clump, imparting a foamy look to the infected nuclei. Small halos were seen surrounding nucleoli, and the nuclei appeared to be swollen (fig. 2).

Twenty-three hours after the virus was placed in cultures, the sheet of cells remained intact. Under low power magnification, however, many cells were seen to contain intranuclear inclusions (fig. 3, 4). The cells in which inclusions had developed were mostly those where cell growth was dense, *i.e.*, the inclusions appeared to have developed in the elongated fibroblast-like cells before they could be seen in the cells which were epithelial in shape. Cells containing inclusions were not in discrete focal arrangement, but in strings which followed the dense, whorled areas. This pattern was identical in all slides. Small groups of cells in the less dense epithelial-like areas also contained inclusions.

There was an increase in the number of nuclei containing inclusion bodies 32 hours postinoculation. These inclusion bodies, as in earlier slides, were located predominantly in the areas of heaviest cell growth. There were a few dead cells but no disruption of the cell sheet.

Many of the cells had died and floated free of the glass, leaving large holes in the cell sheet (fig. 5), at 48 hours after infection. Of the cells remaining attached to the glass, some remained normal or showed only slight nuclear changes; however, many had become shunken and dark, with no cytoplasm visible. At 62 hours, changes were similar to those observed at 48 hours, but more advanced (fig. 6).

In order to determine the progression of changes within individual cells, criteria were established to place changes in four stages, and cells were counted. At each time interval, 500 cells were counted in each of four random fields on each slide. Means of these counts are recorded as percentages of cells seen in each stage (graph 1). As severity of cellular lesions increased, there was some distortion of these percentages because many cells had died, floated free of the glass, and could not be enumerated. This graph, therefore, is only an approximation of numerical relationship of stages of degeneration, but gives an effective over-all picture of the stages through which the cells passed.

Stage I.—These were cells with normal appearing nuclei. They contained no intranuclear inclusion bodies.

Stage II.—The chromatin network had become aggregated into coarse strands, and there was an

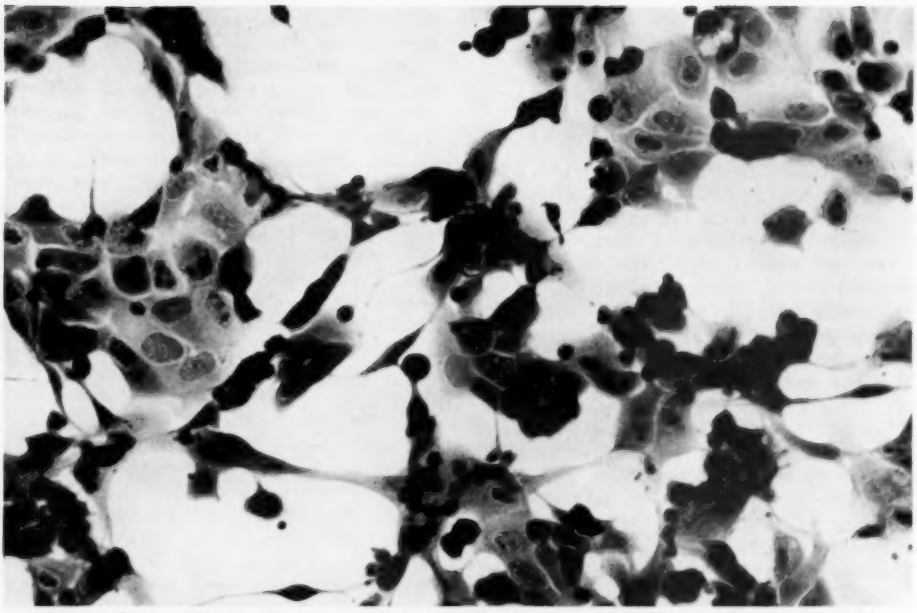


Fig. 5—Canine kidney cells 48 hours after inoculation with virus. Notice gaps in cell sheet where dead cells have fallen away from surface of cover slip. x 375.

Fig. 6—Canine kidney cells 62 hours after inoculation with virus. x 375.

increased density of staining in the center of the nucleus, indicating formation of an inclusion body. This stage was differentiated from stage III by having strands of heavy chromatin, which extended from the edges of the inclusion toward the nuclear membrane.

Stage III.—The nuclei contained dark inclusions with smoother surfaces than in stage II, and a clear halo often surrounded the inclusions. These inclusions usually occupied one half or more of the nucleus. Examples of stages I, II, and III are shown (fig. 4).

Stage IV.—Nuclei were dark and shrunken, with only thin strands of the remnants of cell cytoplasm connecting them to surrounding cells and to the glass surface (fig. 6).

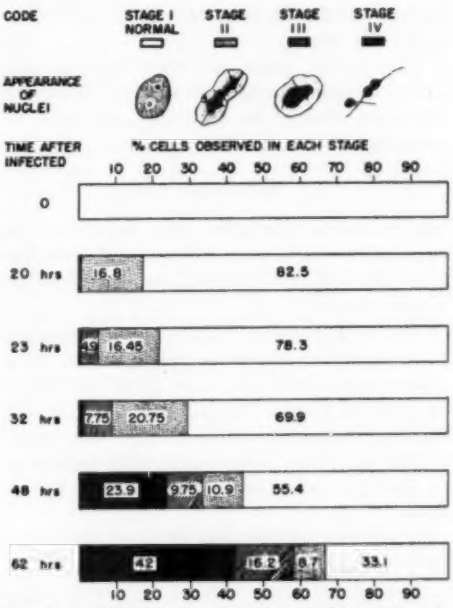
Until the latest stages of degeneration, no specific changes could be seen in the cytoplasm of infected cells. Description of the changes has been limited to those which occurred in the nucleus.

Virus Production.—The concentrations of virus produced by infected cultures are presented (graph 2). Any increase in virus content after the last wash represented virus multiplication within infected cells. It can be seen in the graph that virus level remained low for approximately 20 hours and then rose rapidly, reaching a maximum level about 43 hours postinoculation.

DISCUSSION

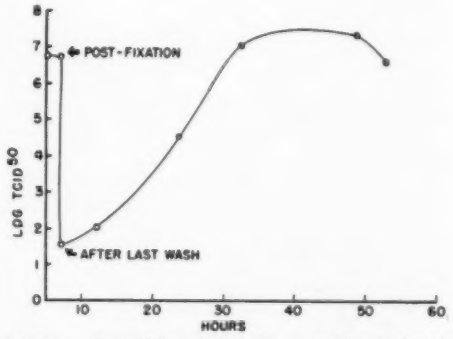
Other authors, Cabasso *et al.*,[1] have pointed out that two cell types are easily differentiated in cultures of canine kidney cells. From examination of these stained cover slip preparations, this could not be entirely substantiated. There was a definite variation in cell shape from round or polyhedral to spindle. However, intermediate shapes were seen between these extremes. Wherever cell growth was sparse, all cells appeared epithelial in type while, in areas of dense growth, the cells appeared more crowded and were elongated, more closely resembling fibroblasts in shape.

In these experiments it was not possible to differentiate any cell type which was not affected by the agent. Even though infectious units were placed on the cells, far in excess of numbers necessary to infect the entire cell population simultaneously, this did not occur. The cells in most dense growth areas appeared to be infected first, but eventually all cells were affected. In the early stages, however, normal cell nuclei could be seen immediately adjacent to infected cells (fig. 4). Slides in advanced stages of degeneration contained some nearly normal cells (fig. 5).



Graph 1—Percentages of canine kidney cells observed to be in each stage of nuclear degeneration at various times following inoculation. Notice that this curve correlates closely to the rate of virus release.

Canine kidney cells infected with ICH virus constitute an excellent approach to basic *in vitro* studies of the host-to-virus relationship. The morphological effects are distinctive, exhibiting profound nuclear changes. The agent itself is easy to handle, being quite resistant to environmental ex-



Graph 2—Quantities of virus (expressed as t.c.i.d.$_{50}$) found in fluids surrounding infected cells. Drop in level at the end of curve probably represents inactivation of infectious particles because of environmental conditions.

tremes. So far as is known, it has no infectivity for man.

Time-lapse, phase-contrast motion pictures, which are planned for future investigations, should be especially effective in studying this cell-virus system.

SUMMARY

Cytological changes are described as they occur in canine kidney cell cultures inoculated with infectious canine hepatitis (ICH) virus.

The first definite change was the appearance of intranuclear inclusion bodies, commencing at about 23 hours after cells were exposed to virus. The number of cells containing inclusions subsequently increased. As the infection progressed, cells were seen to die, and large gaps appeared in the cell sheet.

Estimates of the rate of virus propagation were made by assay of fluids surrounding infected cells at intervals after cells were inoculated.

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Hemangiopericytoma in the Dog

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In 1942, Stout and Murray [6] reported nine vascular tumors in man, in which the principal cell type was thought to be derived from the pericytes of Zimmermann, so-called because the Swiss pathologist of that name had described such cells in the frog in 1923.[8] Although they had no myofibrils, he believed them to be related to smooth muscle, for they had contractile properties and were closely applied to the walls of small capillaries, in which they seemed to function in the regulation of the luminal diameter.[1]

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In a subsequent publication, Stout [5] reported 25 more cases, 24 in man. In the remaining case, a single large tumor, 7.0 by 3.8 cm., was removed from the elbow region of a 7-year-old female Collie. The mass appeared to be encapsulated and had been present for six or seven months. Neoplasms with the morphological characteristics described by Stout have been recognized at the Armed Forces Institute of Pathology as a distinct entity in the dog since 1946,[3] and recently Mulligan [4] has reported 68 cases of similar tumors in the dog. Although there is disagreement as to the exact origin and function of the principal cell type, the structure of the tumor and its behavior in the host leave little room for argument concerning its existence as a distinct entity in the dog.

Materials and Methods

A survey of all the spindle cell tumors in the Registry of Veterinary Pathology at the Armed

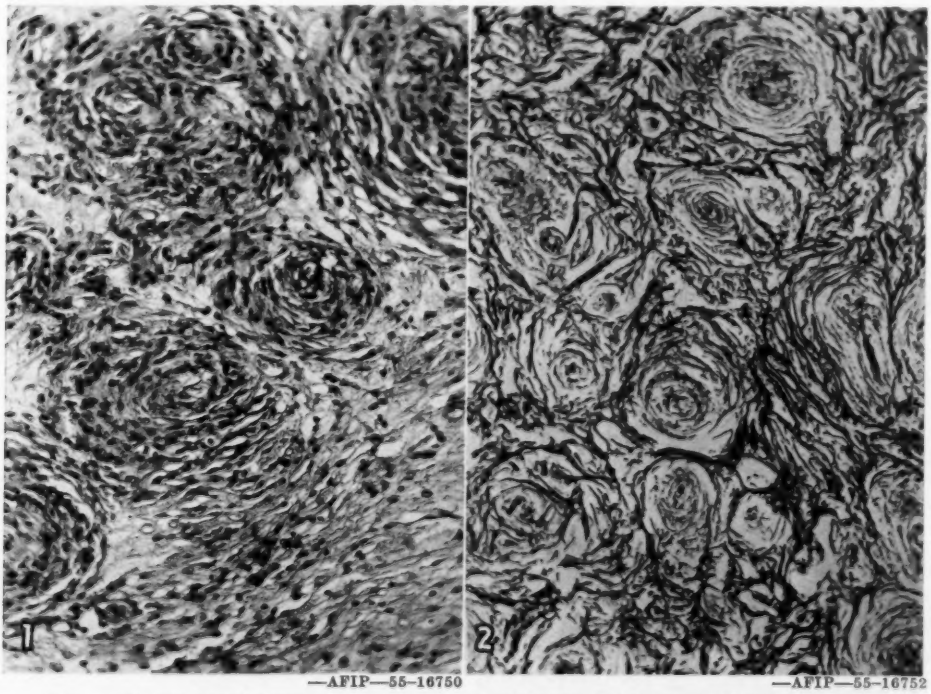

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Fig. 1—Hemangiopericytoma from a 7-year-old miniature Schnauzer. Notice the whorled arrangement of tumor cells around vascular channels. H & E stain; x 160.

Fig. 2—Same tumor as in figure 1. Notice the concentrically laminated fingerprint of the new growth. Wilder's reticulum stain; x 160.

Forces Institute of Pathology was undertaken, with a view to separating those that met the criteria for hemangiopericytoma. The cases reviewed included neoplasms, from all domestic species, diagnosed as neurofibroma, neurofibrosarcoma, neurilemmoma, fibroma, fibrosarcoma, hemangioendothelioma, leiomyoma, and leiomyosarcoma. Sections of all tumors were stained with hematoxylin and eosin, and by the Gomori trichrome [8] and Wilder reticulum [7] methods. On the basis of their morphological features, 34 cases representative of hemangiopericytoma (9%) were retained for further study. All but one of the 34 tumors occurred in the skin and subcutaneous tissues of the dog. A single neoplasm found in the skin of the udder of a 4-year-old Ayrshire cow will be the subject of a separate report.

TABLE 1—Age and Sex Distribution

Age at first occurrence (yr.)	No. of dogs		
	Total	Male	Female
	31 *	21	10
5	1	....	1
6	2	2	....
7	1	....	1
8	5	3	2
9	9	4	5
10	5	5	....
11	1	1	....
12	3	3	....
13	2	1	1
14	1	1	....
15	1	1	....
Average age	9.6	10.0	8.6

* Excludes 1, 9-year-old dog, with sex not reported, and 1 dog with age and sex not reported.

MICROSCOPIC FEATURES

The histological criteria used in selection of the cases were as follows: The characteristic cells were arranged about the lumina of small blood vessels, ranging from occult capillaries to small arterioles. In many areas, the tumor cells were concentrically laminated about vascular spaces with endothelial linings. The tumor cells made up the capillary sheath, with the long axis of the cells perpendicular to the long axis of the vessel (fig. 1, 2). Many of these vascular spaces were open and contained blood; others were empty and collapsed and could be distinguished only when the capillary sheath was delineated by reticulum stains (fig. 3). In some instances, the lumen of the vessel was seen as a tiny smudge of collagenous tissue. The typical whorled or "fingerprint" arrangement of the characteristic cell type was the principal identifying feature of this neoplasm (fig. 2).

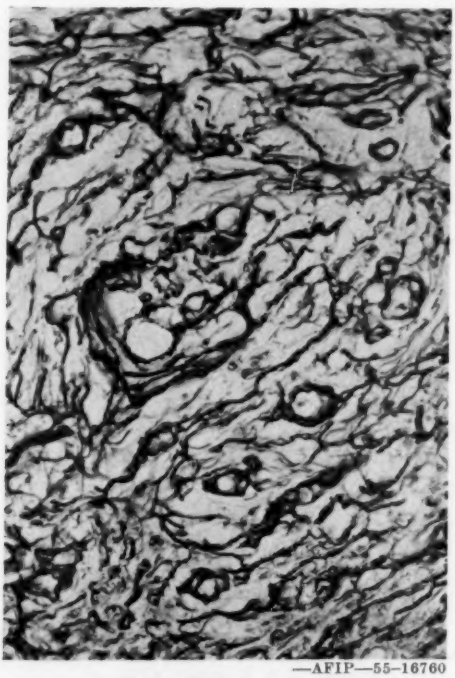

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Fig. 3—Hemangiopericytoma in an Irish Terrier. Reticulum fibers outline the vascular spaces in the neoplasm. Wilder's reticulum stain; x 490.

The type cell of the hemangiopericytoma is unlike the cells of other spindle cell tumors. Although the cells are spindle-shaped, the nuclei are ovoid, with a heavy, coarsely granular, nuclear membrane and a lacy, indistinct chromatin network that

TABLE 2—Postoperative Data (Duration from Initial Operation to Last Report)

	No. of animals
Recurrences	13
One recurrence	7
Less than one year	1
One year	2
Three years	1
Four years	1
Duration unknown	2
Two or more recurrences	4
Three years	1
Four years	2
Six years	1
Metastasis	2
One year (no recurrence)	1
Three years (one recurrence)	1
No recurrence	10
Died immediately after operation	2
Less than one year	2
One year	2
Five years	2
Duration unknown	2
Total	23
No data on subsequent course	10
Total	33

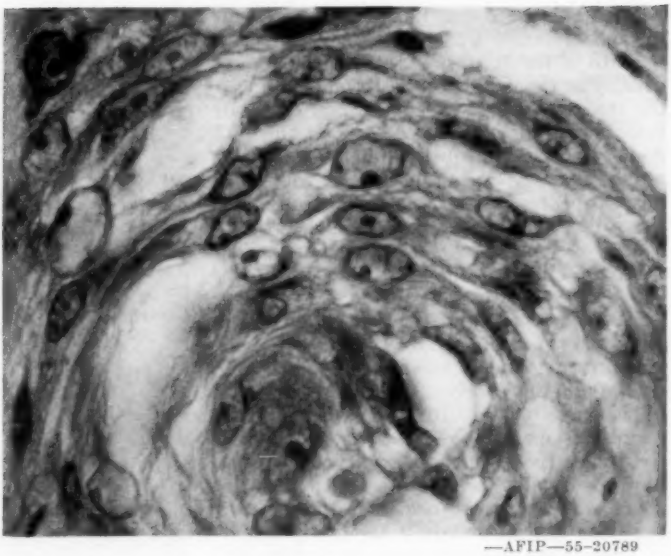

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Fig. 4—A neoplasm from a 12-year-old Terrier. Characteristic ovoid nuclei of typical cells, with marginated chromatin and a single nucleolus. The tumor cells are arranged around an occult vascular space. x 997.

fills the remainder of the nucleus, except for one or two sharply defined, round, centrally located nucleoli (fig. 4). The cytoplasm contains fibrillar processes that may vary greatly in length and thickness (fig. 5) but usually are longer at one pole of the cell.

Perhaps the most significant feature of the canine hemangiopericytoma is the intimate relation between the vascular spaces and the typical cell; so intimate, in fact, as to suggest that the growth of one could not proceed in the absence of the other. The most striking morphological feature for identification and differentiation from spindle cell tumors of other types is the laminated fingerprint arrangement of the typical cells around the vessel outside the vascular endothelium. This differentiates hemangiopericytoma from hemangioendothelioma, in which the growth of neoplastic cells occurs inside the vascular sheath.

Many of the neoplasms are densely cellular, with very little intercellular space and limited amounts of cytoplasm. In others, however, the growth is loose and myxomatous, with the typical cells and growth pattern always present (fig. 6).

CLINICAL FEATURES

After the cases were selected on the basis of histological features, the case records were reviewed for the purpose of discovering any other constant biological features. Without exception, the initial site of the tumor was found to be in the subcutaneous tissues. All but two of the 68 tumors reported by Mulligan were subcutaneous. The majority of neoplasms were firm, yellowish to white in color, and frequently lobate. A few were less firm than others, but none was truly soft. In most cases, the neoplasm was firmly anchored in the deep tissues, with the overlying skin moving freely. Several of the tumors clearly invaded the dermis. Size varied greatly, from 0.6 cm. to 25.0 cm., according to the figure for greatest diameter given in surgical or necropsy protocols. No breed specificity was demonstrated; 22 breeds were represented, with no more than 3 cases in any one known breed.

Certain trends were observed from the clinical data available. Males were predominant, 2:1 (table 1) and, as with most neoplasms, hemangiopericytoma appeared most frequently in older dogs. The youngest dog in the series was a 5-year-old Springer Spaniel and the oldest a 15-year-old Terrier. The average age at first occurrence of the tumor was 10 years (table 1).

FOLLOW-UP STUDIES

Follow-up data were available on 23 animals (table 2). No subsequent history

could be obtained for 10 animals in which the initial tumor was treated surgically.

In 13 of the dogs, the neoplasm recurred, usually at the original site. In one instance, the tumor recurred at the original site and also in several other locations on the abdomen. It is of interest that in only 1 animal was the recurrent tumor known to have appeared in less than one year after the initial operation. In 2 animals, recurrence was one year after surgical removal; in 1, three years, and in 1, four years. In the 4 dogs in which multiple recurrences were seen, none appeared in less than one year after initial removal. In the 2 animals in which metastasis occurred, one year was again the shortest interval before reappearance of the tumor. In these 2, the neoplasm metastasized widely, involving the lungs, liver, and other internal organs, and eventually caused death. However, there was no recurrence in 4 animals for one year or less and in 2 for five years.

These data indicate that, while this tumor usually grows slowly, it can be expected to recur in more than 50 per cent of the dogs treated surgically.

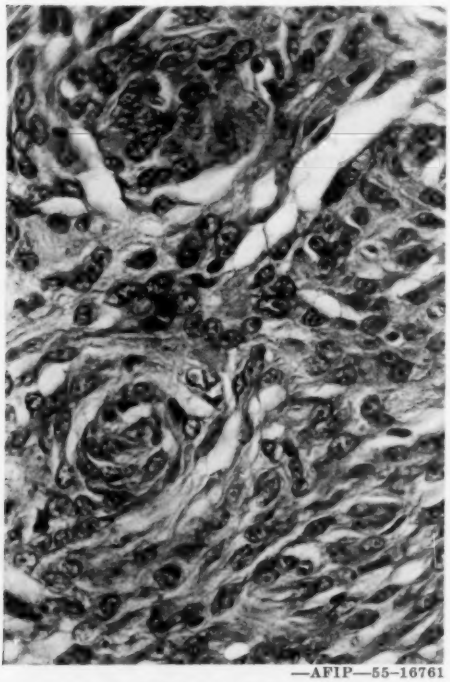

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Fig. 5—Tumor from a 10-year-old Irish Terrier. Uniform ovoid cells form both diffuse and solid areas and are laminated around vascular spaces. H & E stain; x 490.

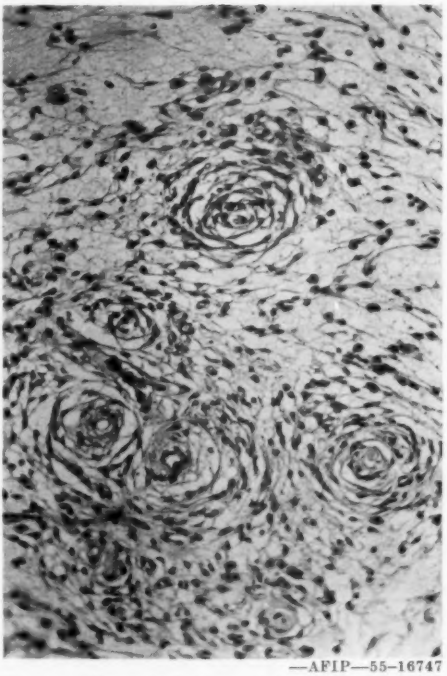

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Fig. 6—Hemangiopericytoma from a 10-year-old Pointer. The cells are less closely packed, but in typical concentric arrangement. H & E stain; x 160.

SUMMARY

Hemangiopericytoma in the dog is a neoplasm with striking repetitive pattern of vascular spaces surrounded by laminated whorls of cells, morphologically distinct from those in other spindle cell tumors. The intimate relation of the typical cells to the vascular spaces, their arrangement around the vessels outside of the endothelial lining, and the characteristic ovoid vesicular nuclei and the spindle-shaped cytoplasm of the cells proper are of cardinal importance in the recognition of this tumor and in differentiating it from other spindle cell neoplasms. This tumor, except in metastatic lesions, appeared only in the skin and subcutaneous tissues in our series.

Originally thought to be rare, hemangiopericytoma is now known to be fairly common among skin tumors of the dog. It may be either benign or malignant, frequently recurs after surgical removal, and may

metastasize widely to cause the death of the animal. Histologically, there is little difference between the benign and malignant forms. Few mitotic figures occur in either form and, although some of the recurrent neoplasms may present a slightly less organized appearance, malignancy is usually determined only upon recurrence or metastasis of the tumor.

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Feline Infectious Anemia. I. Clinical Aspects

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FELINE INFECTIOUS ANEMIA, an acute or chronic disease of domestic cats, is characterized by emaciation, depression, anorexia, high initial temperature, and a macrocytic, hemolytic anemia. It is caused by an organism of the genus Hemobartonella, for which the name, *Hemobartonella felis*, is proposed.

This newly recognized disease was studied in two phases: (I) clinical or spontaneous cases, and (II) experimental cases. The latter will be reported in a subsequent paper.

REVIEW OF LITERATURE

Literature pertaining to blood-parasite diseases of cats is meager. In 1942, Clark [2] described what appeared to be an eperythrozoon infection of the erythrocytes of a South African cat that died from anemia. "Practically all the parasites were ring forms, 0.5 to 1.0 μ in diameter," he stated. No experimental work was carried on by Clark, as only blood and spleen smears were available.

In 1953, Flint and Moss [3] reported briefly on their work in Colorado with an erythrocyte parasite which caused fatal anemia when 0.5 ml. of infected blood was given intraperitoneally to either splenectomized or intact cats. They found that the incubation period varied considerably. Usually, the organisms were found in the peripheral blood smears between postinoculation days 13 and 21, with fatal anemia developing between days 24 and 33.

Flint and Moss found gross and microscopic lesions to be minimal and inconstant. They stressed the pleomorphism of the organisms, which were described as ". . . small, round bodies on and apparently attached to the red cells." In one instance, a cat, used as a donor for blood transfusions to 2 other cats, was later found to be infected. Both recipient cats contracted the disease and died.

Splitter,[12] early in 1956, found the organisms in cats which were selected at random in Kansas. In general, his work paralleled that of Flint and Moss.

Other workers in many parts of the United States have reported cases of the disease. California,[11] Idaho,[4] Utah,[13] Colorado,[10] Nebraska,[6] Minnesota,[9] and Massachusetts [5] are among states in which the infection in cats has been found.

The genus Hemobartonella (fig. 1, 2) is widespread in animals throughout the world and has been reported in many species of rodents,[8, 14] cattle,[7] and dogs.[1] As a group, the organisms are pleomorphic, but with a tendency to appear as cocci, coccobacilli, or short rods, and they often appear in short chains. Ring forms (as commonly seen in eperythrozoon infections) are rare. The organisms are routinely found on or attached to the erythrocytes.

CLINICAL CASES

Over a period of about three years, 30 cats with clinical cases of feline infectious anemia were presented at the Flint Veterinary Hospital in Salt Lake City, Utah. The cats, 28 males and 2 females, had been raised, so far as could be learned, within a radius of 50 miles of the clinic

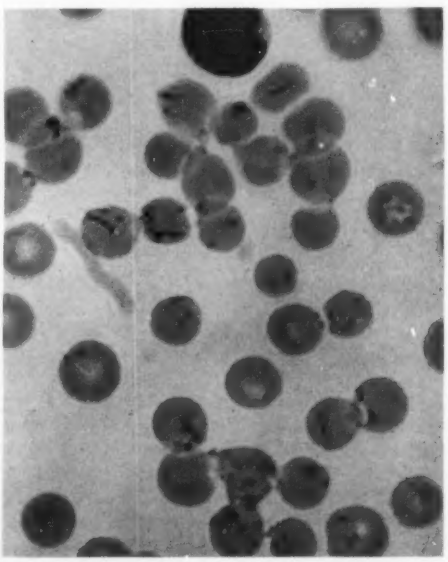

Fig. 1—Both coccoid and rod forms of Hemobartonella felis are apparent on erythrocytes of a 2-year-old cat. Poikilocytosis is present. x 1,400.

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A portion of these data was included in a thesis submitted by Dr. Flint to the Graduate School, University of Minnesota, in partial fulfillment of the requirements for the Ph.D. degree.

TABLE 1—Age Distribution of 30 Cats with Clinical Cases of Feline Infectious Anemia

	Age groups (in years)				
	Under 0.5	0.5 to 1.0	1.1 to 3.0	3.1 to 6.0	6.1 to 12
No. of cats	2	2	22	2	2

and were of mixed breeding, except for 2 Siamese cats.

Fifteen of the cats were moribund when admitted to the hospital. The other 15 were treated by various methods, with emphasis on blood transfusions, arsenical injections, and broad-spectrum antibiotics.

The age incidence (table 1) raised the question of mode of transmission. Some factor, or factors, apparently favored development of the disease (or relapse) shortly after the animals reached sexual maturity.

There were no marked seasonal variations in occurrence, although more cases occurred in the fall, winter, and spring months than in the summer months.

ETIOLOGY

An initial peripheral blood smear (Giemsa's stain) was made from each affected cat. No blood parasites were found in 5 cats, 6 were classified as "suspicious," and *H. felis* was present in 19. Bacillary, coccoid, and occasional ring forms were present. Small "dots" and cocci were attached to the surfaces of erythrocytes. Frequently, they appeared in chains which contained 4 to 8 cocci. When attached to the periphery of the cells, the organisms frequently appeared as rods of considerable length, often with a diameter at least twice that of the coccoid forms. True "circle" or "ring" forms were rarely seen. Organisms were rarely seen free in the plasma and, in these cases, the animal was usually near death.

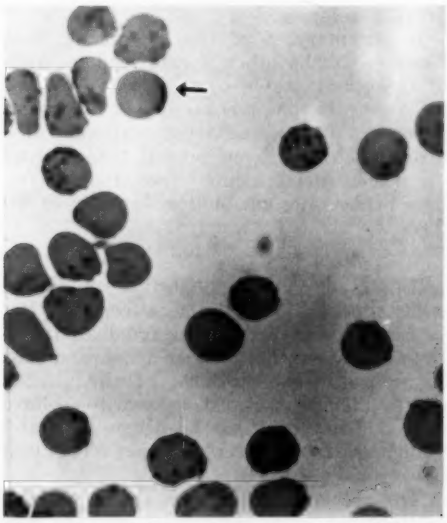

Fig. 2—Coccoid forms of Hemobartonella felis predominate on erythrocytes of this 1-year-old cat. One cell shows rod form attached to periphery of cell (arrow). Both poikilocytosis and anisocytosis are apparent. x 1,400.

Coccoid forms usually had a diameter of 0.2 to 0.3 μ. Rod forms varied in length from 0.7 to 0.9 μ, with an average diameter of about 0.3 μ (fig. 1, 2).

SIGNS OF INFECTION

Weakness, depression, inappetence, rough coats, and marked anemia were present in every clinical case. Six cats were brought to the clinic for treatment of abscesses. Rectal temperatures varied with the condition of the animal on admission. The cats had subnormal temperatures when moribund; cats with chronic cases usually had normal temperatures, while cats with acute or subacute cases had temperatures of 103 to 106 F. (table 3). The presence of icterus was not constant.

Seven cats appeared to be acutely ill, and 23 were listed as having chronic cases; the latter were considered relapses in carrier cats.

HEMATOLOGY

Hemoglobin.—The initial hemoglobin levels of the 30 sick cats were low (table 2); all were under 7.0 Gm./100 ml.

TABLE 2—Initial Hemoglobin Levels in 30 Cats with Clinical Cases of Feline Infectious Anemia

	Hb. (in Gm./100ml.)			
	Under 4.0	4.0 to 4.9	5.0 to 5.9	6.0 to 6.9
No. of cats	19	7	2	2

Erythrocytes.—Erythrocyte counts were correspondingly low, varying from less than 1 million to a high of 5 million, with a mean of 2.25 million cells per cubic milliliter.

Leukocytes.—Initial leukocyte counts on 16 cats were near normal. Moribund animals, where the erythropoietic system was

TABLE 3—Initial Rectal Temperatures in 30 Cats with Clinical Cases of Feline Infectious Anemia (F.)

Cases (No.)	Below 100.0	100.0 to 100.9	101.0 to 101.9	102.0 to 102.9	103.0 to 103.9	104.0 to 104.9	105.0 to 105.9	106.0 to 106.9
Chronic	11	3	3	3	3	0	0	0
Acute	0	0	0	0	1	3	2	1

apparently exhausted, had leukopenia. Those with acute cases, however, had elevated counts. These data are summarized (table 4).

TABLE 4—Initial Leukocyte Counts in 16 Cats with Clinical Cases of Feline Infectious Anemia

	Leukocytes (in thousands)				
	4.50 to 5.99	6.00 to 8.99	9.00 to 12.99	13.00 to 19.99	20.0 to 30.00
No. cases	1	3	5	5	2
Mean	4.60	8.18	11.52	16.38	21.38

General Hematological Signs of Anemia.—Anisocytosis, macrocytosis, diffuse basophilia, and nucleated erythrocytes (normocytes) were nearly always present in peripheral blood smears. Poikilocytosis was not commonly observed.

PATHOLOGICAL CHANGES

Gross Changes.—Anemia was the most prominent gross pathological change in the 21 cats necropsied. Emaciation was advanced in 16 cases, and splenogmegaly and mesenteric lymphnoditis were present in varying degrees in 16. Bone marrow hyperplasia was evident in 20 cats.

Histopathological Changes.—Microscopic lesions were also minimal, and were not of diagnostic importance. In general, both gross and microscopic lesions were considered related to terminal, or secondary conditions, or both.

TREATMENT

Fifteen cats were given treatments of various types. Blood transfusions, arsenical preparations, and antibiotics appeared to give the best results. Sulfonamides and supportive treatment (glucose in saline solution) had no apparent effect on the organisms, although the latter was of value in sustaining patients through critical periods.

Blood Transfusions.—Twenty-eight blood transfusions were given to 17 cats. On the basis of early clinical improvement, it was found that 40 ml. of citrated blood, given intravenously, produced excellent responses in seven instances, fair responses in five, and poor responses in 13 instances.

On the basis of more lasting benefits, it was found that transfusions appeared to be a major factor in the ultimate recovery of 7 cats. In 2 other cases, improvement was temporary, but the cats ultimately relapsed and died. Three or more transfusions, at three-day intervals, were given to each of several cats.

Arsenicals.—Oxyphenarsine hydrochloride (Mepharsen*) was used intravenously on three patients, rather late in the course of the disease, with doubtful results. Later work on experimental cases indicated the drug has considerable merit, although cats appear to be very sensitive to it.

Antibiotics.—Penicillin appeared to have no effect against the organism, but the broad-spectrum antibiotics, particularly oxytetracycline and chloramphenicol, gave good results at an oral dose of 100 mg. twice daily. It was necessary to continue this treatment for at least 18 to 21 days to prevent relapses.

In summary, 8 of the 15 affected cats recovered. The other 7 cats died in spite of treatment (table 5).

The 8 cats that recovered had high temperatures at the time treatment was initiated. Each was given several transfusions of whole blood; they were then given broad-spectrum antibiotics for ten to 20 days.

DISCUSSION

This study has established a set of circumstances which leave no alternative but to assume that cats suffer from an infectious anemia which is often fatal, and that it occurs in many parts of the United States. There is good reason to believe that this condition has been seen by many veterinarians, but has not been recognized or seriously studied.

The comparatively small amount of hematological work performed on cats consists chiefly of leukocyte and differential counts for the diagnosis of panleukopenia.

The signs of feline infectious anemia are

* Parke-Davis and Co., Detroit, Mich.

not diagnostic and the parasites can not be observed in peripheral blood smears at all times.

Factors which substantiate the view that feline infectious anemia is a distinct entity and occurs in many sections of the United States include the following:

1) Small bodies were readily demonstrated on the erythrocytes of anemic cats.

2) Small amounts of blood from infected anemic cats, given orally, intraperitoneally, or intravenously to normal, susceptible cats, produced a clinical condition which appeared identical with that present in the donor cat (to be discussed in detail in a subsequent paper).

3) Pooled blood from adult cats which appeared normal, clinically and hematologically, produced the disease when injected parenterally into healthy cats.

4) Cats, with hemoglobin values as low as 4 Gm./100 ml., with blood smears showing the bodies on a large percentage of erythrocytes, responded favorably to injections of arsenicals.

It is of interest that 22 of the 30 infected cats were between 1 and 3 years of age, the age when male cats begin to show virile tendencies and often engage in fights. Cat bites frequently result in the formation of abscesses. While there is no evidence to prove that the disease is spread by cat bites, it may be significant that the original case, reported in 1953,[3] was presented for treatment of abscesses caused by cat bites, and abscesses were involved in 6 of the 30 clinical cases reported in this study.

It is believed that many mature cats are carriers of the disease, as a result of earlier exposure. The infection presumably lies dormant until some stress situation, such as pregnancy, abscesses, neoplasia, or other debilitating conditions, lowers host vitality and permits the disease to recur in its original virulent form as a severe parasitemia and anemia.

In general, anemia is so pronounced that blood must be given immediately in order to keep the cat alive long enough for other therapeutic agents to become effective. Trial-and-error methods have established 40 ml. as the optimal amount of blood for

TABLE 5—Unsuccessful Treatment of 7 Cats with Feline Infectious Anemia

Case (No.)	Sex	Age	Initial temp. (F.)	Initial Hb. (Gm.)	Treatment	Termination
A-630	Male	3 yr.	102.0	4.4	80 ml. blood day 1; penicillin, 200,000 units, and streptomycin, 0.25 Gm., 5 days; sulfadiazine, 7.7 gr., 5 days.	Apparent recovery; relapsed 40 days later and died.
(Returned to hospital 40 days later)						
A-630	Male	3 yr.	102.2	4.4	60 ml. blood on days 6, 69, and 83; chloramphenicol, 200 mg. for 9 days; oxyphenarsine hydrochloride, 80 mg. on day 13; 45 mg. on days 17 and 21.	Died 87 days after apparent recovery; Hb. was 10.5 Gm. on day 46.
5449	Male	1.5 yr.	98.0	Under 4.0	80 ml. blood day 1; penicillin, 300,000 units for 3 days; liver extract, 1 unit, 3 days.	Died on day 4.
5800	Male	2 yr.	101.4	Under 4.0	Glucose solution, 5%, i.v., 100 ml. twice daily for 5 days; penicillin, 300,000 units 5 days.	Died 30 days later; poor condition.
19344	Male	2 yr.	103.0	4.5	20 ml. blood on days 3 and 6; penicillin, 300,000 units, 5 days; liver extract, 1 unit, 5 days.	Died on day 6.
9250	Male	3 yr.	102.5	6.0	40 ml. blood on days 1, 3, and 6; tetracycline, 200 mg. daily for 7 days.	Died on day 8.
6978	Male	6 yr.	103.2	Under 4.0	70 ml. blood, day 1; 50 ml. blood, days 13 and 20; oxyphenarsine hydrochloride 20 mg. i.v., on days 5 and 8; chloramphenicol, 100 mg., 11 days; liver extract, 1 unit, 11 days.	Died on day 23. Hb. never over 6.0 Gm.
8367	Male	1.5 yr.	100.0	Under 4.0	30 ml. blood day 1; 40 ml. blood on days 3, 5, and 56; penicillin, 300,000 units for 6 days; oxytetracycline, 200 mg. for 20 days.	Died 57 days after apparent recovery; Hb., 11.0 Gm. on day 34.

transfusion of an adult cat. Often, transfusions must be repeated at two- or three-day intervals, as parasitic destruction of erythrocytes is rapid and continuous.

Flint and Moss [3] and Splitter [12] have shown that many normal-appearing cats are latent carriers of the infection. Thus, it is apparent that blood of some donors will be infective and their blood constitutes a potential danger to the recipient. This is a risk which must be taken, however.

Evaluation of treatment of clinical cases is usually difficult, and was especially so in this series of 30 cases, as some were chronic, some acute, and some moribund. Methods of treatment improved with experience, so it seems reasonable to suppose some of the earlier cats with fatal cases might have been saved with methods used later in the investigation.

Relapsed Cases.—The problem of relapses, when the animal appears completely recovered, is one that needs further study. Two of the unsuccessfully treated cats (table 5) made excellent improvements for varying lengths of time, and then relapsed and died.

Cat A-630 had a normal hemoglobin, 10.5 Gm./100 ml., and appeared clinically well on the forty-sixth day following treatment, yet relapsed and died on the eighty-seventh day, despite energetic treatment.

Similarly, Cat 8367 had a hemoglobin level of 11.0 Gm. and appeared clinically normal on the thirty-fourth day, but relapsed and died on the fifty-seventh day. This case is especially interesting, as it is the only relapse recorded (in either naturally occurring or experimental cases) after treatment for 20 days with a broad-spectrum antibiotic.

SUMMARY

1) Feline infectious anemia is an acute or chronic blood parasite disease characterized by emaciation, depression, anorexia, and a high initial temperature, with rapid development of a macrocytic, hemolytic anemia which produces a characteristic blood picture. Data on 30 clinical cases are reported in this phase of the investigation.

2) It is believed the etiological agent is a member of the genus Hemobartonella. The specific name, *Hemobartonella felis,* is proposed.

3) The organisms appear as small cocci or rods on the erythrocytes of blood smears stained with Giemsa's or Wright's stains. The organisms are not always visible in peripheral blood smears. Frequently they are present for a day or two, disappear for a few days, and then reappear.

4) Diagnosis is based on many factors which are not pathognomonic. Anisocytosis, macrocytosis, and immature erythrocytes are always present in the peripheral blood smear. The appearance of organisms on the erythrocytes is diagnostic, but their absence does not warrant a negative diagnosis. A hemoglobin value of 7 Gm./100 ml., or less, would suggest that the condition was feline infectious anemia.

5) The method of transmission has not been determined.

6) The mortality varies greatly. Many cats apparently overcome initial infections and become latent carriers of the disease. Stress conditions presumably produce relapses in some carriers.

7) Comparatively few therapeutic agents have been tried in treating the disease. Blood transfusions are of great value in maintaining the patient, and injectable arsenicals and broad-spectrum antibiotics offer the best known method of treatment.

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The Effect of Chlortetracycline Treatment of Turkeys Affected with Ornithosis

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VARIOUS FORMS of medication have been used under field conditions to treat ornithosis in turkeys.[1,6,7] These treatments were based on knowledge obtained from treating human beings and psittacine birds affected with psittacosis.[3,8-10,13,14] A search of the literature revealed no reports of controlled experiments on the treatment of turkey ornithosis; however, the use of chlortetracycline (CTC) as a prophylactic agent has been reported.[5,12]

The purpose of this paper is to report the efficacy of chlortetracycline as a therapeutic agent against ornithosis after the disease has been established in turkeys.

MATERIALS AND METHODS

Four separate experiments were conducted at different times. In the first two experiments, 3-week-old poults were used; in the last two experiments, mature turkeys were used.

Each turkey was inoculated intratracheally with 0.25 ml. of amnioallantoic fluid of chicken embryos infected with fifth passage of Jo strain ornithosis virus. At various intervals after inoculation, the turkeys were given feed containing different levels of CTC for two or three weeks. The CTC was incorporated in the feed at the rates of 100, 200, 400, and 800 Gm. per ton, and biological assays by the manufacturer revealed the CTC content to be within standard tolerance limits. After the treatment period, the turkeys were given unmedicated feed for three days before virus isolation attempts were made, because CTC may have a short-time residual effect in tissues.[2]

Each turkey which died was necropsied and the lesions recorded. At the termination of the feeding period, the surviving turkeys were killed, lesions recorded, and the spleen of each turkey was assayed for the presence of the virus. Each spleen was emulsified in nutrient broth, and 0.2 ml. of the suspension was inoculated into the allantoic cavity of each of 5, 7-day-old chicken embryos. Embryo mortality patterns and results of microscopic examinations for Levinthal-Cole-Lillie bodies in embryonic fluids were the criteria for determining the presence of ornithosis virus. Two blind passages in chicken embryos without recovering the agent were considered negative. This applied to all experiments; variations concerning number of turkeys, dosage of antibiotic, and duration of treatment are given.

EXPERIMENTAL PROCEDURE

Experiment 1.—Groups (13) of 10 Broad-Breasted Bronze poults were used (table 1). Birds in all groups except the last were inoculated with virus. Those in groups 1 and 2 served as untreated control birds. Three days postinoculation, those in groups 3, 4, 5, and 6 were given feed containing 100, 200, 400, and 800 Gm. of CTC per ton, respectively. Those in groups 7, 8, 9, and 10 were given the same treatment four days after

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TABLE 1—The Results of Feeding Chlortetracycline in an All-Mash Ration for Two Weeks to 3-Week-Old Poults Infected with Ornithosis

Group No. (10 poults each)	Gm. of CTC/ton	Day therapy started (post-inoculation)	Mortality (days deaths occurred)	Virus recovery	Survivors (killed after 2 weeks)
1	(Inoculated-untreated)		10 (5–11)	....	*
2	(Inoculated-untreated)		9 (4–10)	....	*
3	100	3	2 (6–18)		6/8
4	200	3	0		6/10
5	400	3	0		8/10
6	800	3	0		2/10
7	100	4	5 (4–9)		2/5
8	200	4	2 (5)		5/8
9	400	4	2 (4–15)		4/8
10	800	4	2 (5–10)		2/8
11	400	5	2 (5–6)		2/8
12	800	5	1 (5)		2/9
13	(Isolated controls)		0		.....

* Viremia detected daily, second to tenth days.

TABLE 2—The Results of Feeding Chlortetracycline in an All-Mash Ration for Three Weeks to 3-Week-Old Poults Infected with Ornithosis

Group No. (10 poults each)	Gm. of CTC/ton	CTC, i.p.* injection	Day therapy started (post-inoculation)	Mortality (days deaths occurred)	Virus recovery	Survivors (killed after 3 weeks)
1		(Inoculated-untreated)		10 (5–12)		.....
2	100	.....	3	1 (4)		4/9
3	100	10 mg.	3	1 (4)		4/9
4	200	.....	3	1 (1)		0/9
5	200	10 mg.	3	1 (13)		0/9
6	400	.....	3	0		0/10
7	400	10 mg.	3	0		0/10
8	800	.....	3	0		0/10
9	800	10 mg.	3	0		0/10
10	400	.....	4	1 (5)		0/9
11	400	10 mg.	4	1 (17)		0/9
12	800	.....	4	1 (23)		0/9
13	800	10 mg.	4	0		0/10
14		(Isolated controls)		0		.....

* i.p. = intraperitoneal.

inoculation. The poults in groups 11 and 12 were given feed containing 400 and 800 Gm. of CTC per ton, respectively, five days postinoculation. All treatment periods were of two weeks' duration. Group 13 served as a control group for feed consumption and weight gains.

The poults in groups 1 and 2 were marked and, for ten days, a blood sample was taken from a different poult in each group. Each blood sample was assayed for virus by inoculating 0.2 ml. via the allantoic cavity into each of 5, 7-day-old chicken embryos.

Experiment 2.—Groups (14) of 10 Broad-Breasted Bronze poults were used (table 2). Birds in all groups except the last were inoculated with virus. Those in group 1 served as untreated control turkeys. After three days, the poults in groups 2 and 3 were given feed containing 100 Gm. of CTC per ton; groups 4 and 5, 200 Gm. per ton; groups 6 and 7, 400 Gm. per ton; groups 8 and 9, 800 Gm. per ton. After four days, those in groups 10 and 11 were given feed containing 400 Gm. of CTC per ton and those in groups 12 and 13, 800 Gm. per ton. The treatment periods were of three weeks' duration. In addition to CTC in the feed, each turkey in groups 3, 5, 6, 9, 11, and 13 was injected intraperitoneally with 10 mg. of CTC in sesame oil, simultaneously with the beginning of the feeding of CTC. Group 14 served as the control group for feed consumption and weight gains.

Experiment 3.—Adult, Broad-Breasted Bronze female turkeys (40) were allotted to groups (table 3) and were inoculated with ornithosis virus. The 10 turkeys in group 1 served as untreated control birds. The 15 turkeys in each of groups 2 and 3 were given feed containing 200 and 400 Gm. of CTC per ton, respectively. Therapy was started seven days after inoculation and was continued for three weeks.

Experiment 4.—Adult Beltsville male and female turkeys (60) were allotted to three equal groups (table 4) and all birds were inoculated with virus. Those in group 1 served as untreated control turkeys. Those in groups 2 and 3 were given feed containing 200 and 400 Gm. of CTC per ton, respectively, seven days after inoculation and treatment was continued for three weeks. However, after two weeks, half of the turkeys in each group were killed and assayed for virus; the remaining turkeys were killed and assayed after three weeks.

TABLE 3—The Results of Feeding Chlortetracycline in an All-Mash Ration for Three Weeks to Adult Turkey Hens Infected with Ornithosis

Group No.	No. hens	Gm. of CTC/ton	Day therapy started (post-inoculation)	Mortality (days deaths occurred)	Virus recovery	Survivors (killed after 3 weeks)
1	10	(Inoculated-untreated)		1 (13)		0/9
2	15	200	7	0		0/15
3	15	400	7	1 (13)		0/14

RESULTS

In experiment 1, the mortality was 95 per cent in the untreated controls, and viremia was detected daily from the second to the tenth days in these birds. No birds died as a result of infection in groups 4, 5, 6, and 13. Some mortality occurred in all other groups. Virus was recovered from some of the survivors of each infected group.

In experiment 2, the mortality, except in group 1, was slightly less than in experiment 1. Virus was recovered from some of the survivors in groups 2 and 3, but not from survivors of other treated

TABLE 4—The Results of Feeding Chlortetracycline in an All-Mash Ration for Two and Three Weeks to Adult Turkeys Infected with Ornithosis

Group No. (20 turkeys each)	Gm. of CTC/ton	Day therapy started (post-inoculation)	Mortality (days deaths occurred)	Virus recovery / Survivors	
1	(Inoculated-untreated)		2 (13–15)	2/9	(killed 24 days after inoculation)
				0/9	(killed 31 days after inoculation)
2	200	7	0	0/10	(killed after 14 days' therapy)
				0/10	(killed after 21 days' therapy)
3	400	7	0	0/10	(killed after 14 days' therapy)
				0/10	(killed after 21 days' therapy)

groups. There was no demonstrable benefit from the injections of CTC.

In experiment 3, the mortality was low; 1 hen in group 1 and 1 hen in group 3 died. Virus was not recovered from any of the survivors when killed. In group 1, 31 days had elapsed from the time of inoculation when the 9 survivors were killed and assayed.

In experiment 4, 2 males died in group 1, but no deaths occurred in other groups. In group 1, virus was recovered from 2 of the 9 turkeys killed 24 days after inoculation. Untreated survivors killed after 31 days failed to yield virus. Virus was not recovered from any turkeys in the treated groups.

The lesions observed in birds which died during the two experiments involving 3-week-old poults were the same as those previously reported for an acute infection.[4] When the survivors were killed, few lesions were observed; aerosacculitis and peritonitis were the most constant findings. Usually, the higher the level of drug, the fewer the lesions. In some of the survivors, especially those given medicated feed for three weeks, there were no active caseofibrinous lesions. However, there were fibrous adhesions within the pericardial sac and raised fibrous areas on the capsule of the liver, representing organization of a previous fibrinous exudate. There was no correlation between the type of lesion and the recovery of virus in either experiment.

In the experiments with adult turkeys, pericarditis was the most striking lesion. While some fibrinous exudates were observed within the pericardial sacs of a few birds, the lesions observed were usually undergoing organization resulting in fibrous adhesions (fig. 1, 2). There were, essentially, no differences between the lesions observed in the untreated and treated turkeys which were killed.

DISCUSSION

By the use of 3-week-old poults, these investigations served as a pilot study for experimentation with adults. Ornithosis has not been known to occur naturally in

Fig. 1—Heart of a turkey with ornithosis, after treatment, showing heavy accumulation of fibrinous exudate undergoing organization on the epicardium.

poults younger than 10 weeks of age; therefore, it is speculative whether information derived from these studies is applicable to the control of ornithosis under field conditions.

It was of interest that, even though CTC was administered to poults for two weeks, in concentrations as high as 800 Gm. per ton, ornithosis virus was recovered from some of the survivors. However, when the treatment period was extended to three weeks, virus was recovered from only those on the 100-Gm.-per-ton level. It has previously been shown that poults are more susceptible to ornithosis than adult turkeys.[4] In these studies, the response to therapy, the mortality, presence of lesions, and recovery of virus substantiated this observation.

The use of feed containing 200 Gm. of CTC per ton for two weeks apparently was effective in treating ornithosis in adult turkeys. It is important that the CTC be incorporated in an all-mash ration. Our findings indicate that the responses of infected turkeys to two days of this treatment were remarkable.

Fig. 2—Heart of a turkey with ornithosis, after treatment, showing fibrotic areas (arrows) on surface of epicardium where the pericardium was adhered to the epicardium.

In controlled experiments, all birds can be infected uniformly at the same time, but the rate of infection will vary under field conditions. For treatment to be effective in a farm flock, medication should be given for three weeks at the rate of 200 Gm. of CTC per ton since, in a recent trial with naturally infected turkeys, virus could be isolated from tissues of 1 bird after two weeks on feed containing 200 Gm. of CTC per ton.[6]

No absolute criterion is available for predicting whether ornithosis virus may be recovered from a given type of lesion; yet, when lesions become fibrous and adhesions develop, it would seem that the infection has been largely suppressed or entirely overcome. Virus could be recovered from approximately 25 per cent of the untreated adult turkeys 24 days after inoculation, but from none after 31 days, thus indicating that time plays a prominant role in the elimination of the virus. Page[11] reported slightly longer periods of time for recovery of virus, depending on the route of inoculation used in the turkeys.

Chlortetracycline appears to reduce toxemia and viremia and to prevent mortality.

SUMMARY

Chlortetracycline (CTC), when fed for two weeks in an all-mash ration at levels of 100, 200, 400, and 800 Gm. per ton, did not aid in the elimination of ornithosis virus from infected 3-week-old poults. When similar treatment was given for three weeks, feed containing 100 Gm. of CTC per ton was ineffective, but concentrations of 200 Gm. or greater were effective.

Ornithosis virus was not recovered from experimentally infected adult turkeys that were treated for two or three weeks with feed containing 200 or 400 Gm. of CTC per ton. Virus was recovered from untreated adult turkeys 24 days after inoculation, but not after 31 days.

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Propagation of the Lapinized-Avianized Strain of Rinderpest Virus in the Culture of Chicken Embryo Tissue

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THE RECENT developments in the field of tissue cultures of various viruses inspired us to study and adopt some of these techniques for the propagation of rinderpest virus and to devise a new laboratory means by which biological and immunological investigations of this virus may be rendered easy and inexpensive. Other stimuli were the development of the avianized strains of this virus,[2,3,8,9] which propagate vigorously in chicken embryos, and the progress in the technique of the complement-fixation (CF) test,[6] which is now a reliable diagnostic means in chicken embryos infected with those strains.

This paper describes a method of propagating the lapinized-avianized (LA) virus[3] in chicken embryo tissue, in combination with the application of the CF test for diagnostic purposes.

Takematsu and Morimoto[10] reported the successful propagation and subsequent passage of the lapinized strain of rinderpest virus in a culture of normal rabbit lymph node tissue. No cytopathogenic activity was manifested. The virus propagation was recognized by titrations in rabbits. In a preliminary paper, Plowright and Ferris[7] reported that their bovine strain of virulent rinderpest virus multiplied in monolayers of bovine embryonic kidney cells and produced characteristic cytopathogenic changes, and that kidney-passaged virus destroyed bovine fibroblasts grown from embryonic skin-muscle tissue and prepuberal testis.

MATERIALS AND METHODS

Seed Virus.—The virus seeded to tissue culture was the lapinized-avianized (LA) strain of rinderpest virus[2,8] of the AE393 to AE506 passage level. The virus source was mostly the spleen and partly the body of the chicken embryos, with head and legs severed, and the virus titer (EID) of such seed virus was usually 10^{-6} and 10^{-4} or 10^{-5} per 0.05 Gm., respectively, in embryonating eggs by intravenous inoculation.

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Tissue Fragments.—Tissue fragments were collected from normal chicken embryos. The body, from which the head and legs had been removed, was floated in phosphate-buffered saline solution and minced, in the solution, with scissors, into fragments of about 1 cmm. The fragments were sedimented by light centrifugation and washed with fresh buffered saline solution. The sedimented tissue fragments were measured with a pipette.

Culture Medium.—The basic culture medium was Tyrode's solution containing 100 to 200 units of penicillin and 0.5 to 1.0 mg. of streptomycin per milliliter. In the latter part of the study, normal horse serum was usually added in a proportion of 1:10.

Culture Flasks and Tubes.—Three types of ordinary vaccine containers were used as culture flasks. The type A flask was 27 mm. in inside diameter, with a capacity of 20 ml.; type B flask was 33 mm. in inside diameter and capable of holding 50 ml.; type C flask was of the same capacity as type B, but was 47 mm. in inside diameter. The three types of flasks were used for cultures with 3, 4, and 9 ml. of medium, respectively, so as to make the depth of culture medium uniformly about 5 mm., regardless of the type of flask. Every flask was stoppered with a cotton plug.

In some experiments, in which the roller tube culture was examined, ordinary test tubes, 17 by 180 mm., stoppered with a cotton plug, were used.

Culture.—Using the culture flasks, a simple suspension method, essentially similar to the Maitland method, was used. The tissue fragments were measured into each flask. The culture medium to which the virus had been preliminarily added was poured over the tissue fragments. The flask was stoppered with a cotton plug and slightly agitated, so as to spread the tissue fragments all over the flat bottom, then kept in an incubator at 35.5 or 38.0 C. without rotation or change of medium. The amount of culture medium contained in a flask was definite for a given type of flask. The amount of tissue fragments was calculated in a percentage to the amount of medium.

In some experiments, a roller tube method which had been recommended by Hayashi[1] for the culture of Newcastle disease virus, and by Nakamura and Hayashi[5] for the culture of hog cholera virus was tried comparatively. Test tubes were used instead of flasks, and inserted along the inside wall of each was a narrow rectangular piece of Japanese rice paper, approximately 25 by 45 mm. The tubes were then sterilized. Before use, about 0.5 ml. of Tyrode's solution was dropped into the

tube to fix the paper inside the wall, its lower lip nearly reaching the bottom. Culture medium, virus, and tissue fragments were mixed in another vessel, and 3 ml. of the mixture was placed in each test tube. After being stoppered, each tube was held obliquely and rotated slowly by hand, to fix the tissue fragments on the rough surface of the paper piece, then mounted on a rotating tube holder, placed in an incubator, and culture was made under seven rotations per hour.

Every culture was made, either by the suspension method or by the roller tube method, in a group of two or more flasks or tubes, and titers of virus and CF antigen were usually determined with a pooled material from these flasks or tubes.

Titration of Virus.—The virus titration was performed by the method described by Nakamura *et al.*[4] Twelve- or 13-day embryonating hen's eggs were inoculated intravenously with 0.05 ml. of tenfold serial dilutions of a given sample. After five days' reincubation, embryonic spleens were collected by groups and tested on their CF antigen content. The virus titer or embryo infective dilution (EID) was represented by the limit dilution 10^{-x} of the sample which produced demonstrable antigen in the embryonic spleens.

Demonstration of CF Antigen.—The CF method was applied to the demonstration of specific antigen, either in embryonic spleens or in cultured tissue fragments. The antigen to be tested was a 1:20 saline extract of embryonic spleens or a 1:10 saline extract of tissue fragments. The embryonic spleen antigen was either an extract from dried tissue or a boiled extract from fresh tissue. Both were equally effective. The tissue culture antigen was a boiled extract from fresh tissue. The serum of cattle hyperimmunized with the bovine virus was the source of antibody and the tests were carried out in combination with a normal bovine serum as control.

Complement-fixation was by the simplified micro-TRC method, similar to that described by Nakamura *et al.*[6] Definite amounts of antigen, serum, and complement were mixed in a test tube and left at 1 to 4 C. overnight. The complement, which remained active after exposure to the antigen and antibody (residual complement), was then titrated by diluting the mixture serially in four test tubes and by adding sensitized blood cells. The actual test was composed of two series. The residual complement was titrated after exposure to the antigen and immune serum in the first series, and after exposure to the antigen and normal serum in the second series. The results were read in percentage hemolysis. From the data obtained, the amount of blood cells which remained undestroyed as a consequence of specific fixation of complement was expressed by percentage of the total amount of blood cells which were hemolyzed in the control series. This percentage represented the degree of specific reaction or the fixation titer (FT). The standard for judging positive or negative reaction was as follows:

FT	*Judgment*
⩽ 10	Negative
11 to 20	Doubtful
21 to 100	Positive

For diagnostic purposes, the test was made only with the original antigen, *i.e.*, the 1:20 extract of embryonic spleens or the 1:10 extract of cultured tissue fragments. The titration of antigen was carried out with each of twofold serial dilutions of the antigen, to determine the limit dilution of antigen giving a positive result. The antigen titer (AgD) was represented by the limit dilution which was calculated with the starting amount of tissue, e.g., when a positive reaction was manifested only with the original antigen of cultured tissue fragments, the antigen titer was 1:10.

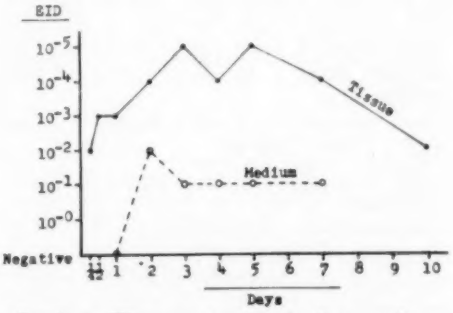
GENERAL VIEW OF VIRUS PROPAGATION

For a general view, the virus was propagated in tissue cultures under such provisional conditions as described below, and periodically titrated with both tissue fragments and medium.

Seed virus—spleen virus having EID 10^{-6} seeded in a dilution of 10^{-3}.
Age and proportion of tissue—5-day-old, 2 per cent.
Medium—Tyrode's solution.
Type of culture—suspension culture in type A flasks.
Incubation temperature—35.5 C.

Three experiments were carried out, the results of which were essentially the same (graph 1). The drop in virus titer in the flask was obvious for the first six hours, after which the titer in the tissue fragments was elevated to the maximum, *i.e.*, 10^{-5}, in three days. After about two days, the virus decreased gradually. Through the entire course of culture, virus was poorly elusive into the medium, its titer being 100 to 10,000 times lower in the medium than in the tissue.

According to Nakamura *et al.*,[4] the LA



Graph 1—Virus propagation in tissue culture.

virus inoculated into embryonating eggs propagated in almost every part of the embryonic body. The highest virus titer, which was usually EID 10^{-4} or 10^{-5}, was reached three days after intravenous inoculation of the virus. The virus in the tissue culture behaved similarly to that in the embryonating egg, and there was little doubt about the true propagation of the virus in the tissue culture.

CULTURE CONDITIONS

Age of Chicken Embryos Which Provide Tissues.—Three experiments were performed to investigate the influence of the age factor of chicken embryos which provide the tissue material. In each experiment, three groups of cultures were prepared from tissue fragments of 5-, 9-, and 12-day-old chicken embryos.

Seed virus—spleen virus in a dilution of 10^{-4} or 10^{-3}.
Proportion of tissue—5 per cent.
Medium—Tyrode's solution or 10 per cent horse serum.
Type of culture—suspension culture in type C flasks.
Incubation—five days at 35.5 C.

In the first two experiments, the virus titer in the cultured tissue was ten times higher in the group of 12-day-old embryos (EID 10^{-5}) than in the other groups of 5- and 9-day-old embryos. The CF antigen was not tested. In the last experiment, the virus titer was the same level for all three culture groups (EID 10^{-6}), but the antigen titer was remarkably higher in the group of 12-day-old embryos (AgD 40) than in the other two groups (AgD 10 in both).

Proportion of Tissue Fragments to Medium.—The next step was to determine the adequate proportion of tissue fragments to the medium in which the highest virus titer of the tissue might be reached. In the three experiments carried out for this purpose, several groups of cultures were prepared from tissue fragments, in amounts from 1 to 20 per cent of the medium.

Seed virus—spleen virus in a dilution of 10^{-3}.
Age of tissue—nine days.
Medium—Tyrode's solution.
Type of culture—suspension culture in type C flasks.
Incubation—five days at 35.5 C.

Cultures containing 5 per cent tissue fragments gave the virus titers of the highest level (EID 10^{-4}, 10^{-5}, and 10^{-5}). An excess of tissue fragments obviously checked the propagation of virus, and cultures containing 20 per cent tissue fragments gave the lowest virus titers (EID $>10^{-2}$, 10^{-2}, and 10^{-3}).

Method of Culture.—The roller tube method was compared with the ordinary suspension method, using the type A flask, in three experiments.

Seed virus—spleen virus in a dilution of 10^{-3}.
Age and proportion of tissue—nine days old; 5 per cent.
Medium—Tyrode's solution.
Incubation—five days at 35.5 C.

The results of virus titrations were never better with the roller tube method. In two of three experiments, propagation of virus was more prominent in the cultures made by the suspension method (EID 10^{-5}, 10^{-4}, and 10^{-4}) than in those by the roller tube method (EID 10^{-4}, 10^{-4}, $>10^{-4}$).

Addition of Serum to Medium.—The effect of adding serum from various animal species to the medium was studied, with regard to individuality and concentration. Chicken, rabbit, and horse serums, all in 10 per cent suspensions in Tyrode's solution, were compared with one another and with Tyrode's solution containing no serum. Nine small experiments were carried out for this purpose.

Seed virus—spleen virus in a dilution of 10^{-2} to 10^{-4}.
Age of tissue—nine or 12 days old.
Proportion of tissue—2 or 5 per cent.
Type of culture—suspension culture in type A or C flasks.
Incubation—five days at 35.5 or 38.0 C.

Virus or antigen, or both, produced in the tissue were titrated with each group of cultures. Throughout the experiments, virus titers in the cultures with serum (EID 10^{-5} or 10^{-6}) were constantly ten or more times higher than those in the cultures without serum (EID 10^{-4}). The production of the CF antigen was remarkably more prominent in the former group (AgD 20 to 320) than in the latter (AgD 10 to 160). No definite differences were manifested among chicken, rabbit, and horse serums.

Individual variations in the effect on virus propagation were then examined in two experiments on four and ten serums from normal horses.

Seed virus—spleen virus in a dilution of 10^{-3}.
Age and proportion of tissue—12 days old; 5 per cent.

Medium—10 per cent horse serum.
Type of culture—suspension culture in type C flasks.
Incubation—five days at 35.5 or 38.0 C.

In these experiments, only antigen was titrated with each culture. There were no individual differences among the horse serums used in each experiment. All four cultures in one experiment uniformly gave an AgD of 1:160 and all ten cultures in the other experiment gave an AgD of 1:40.

Finally, an adequate concentration of horse serum was determined, in two experiments, by adding horse serum to Tyrode's solution in varying proportions of 0, 1, 5, 10, 30, and 100 per cent.

Seed virus—spleen virus in a dilution of 10^{-3}.
Age and proportion of tissue—12 days old; 5 per cent.
Type of culture—suspension culture in type C flasks.
Incubation—five days at 38 C.

There were some irregularities in the antigen titers and it was difficult to give a precise criterion. Nevertheless, it was inferred that our provisional choice of 10 per cent horse serum was correct since, in both experiments, the tissue cultured in this serum concentration gave the highest AgD (80 in both experiments).

Incubation Temperature.—Two incubation temperatures, 35.5 C. and 38.0 C., were compared in five experiments.

Seed virus—spleen virus in a dilution of 10^{-4} or 10^{-3}.
Age and proportion of tissue—nine or 12 days old; 5 per cent.
Medium—10 per cent horse serum.
Type of culture—suspension culture in type C flasks.
Incubation period—five days.

Throughout the five experiments, the production of antigen was stronger, or at least on the same level, in the cultures incubated at 38.0 C. (AgD 40, 80, 80, 160, and 160) than in those incubated at 35.5 C. (AgD 20, 40, 80, 80, and 160).

Standard Culture Conditions.—Based on the knowledge obtained in the preceding experiments, the tissue culture, for the purpose of propagating the LA virus, was standardized.

Type of culture—suspension culture in type B flasks; a pool from three flasks of this type is sufficient for the CF test.
Age and proportion of tissue—0.2 ml. (5% of the medium) of tissue fragments from 12-day-old chicken embryos for each flask; the use of older embryos should be avoided because of thick plumage.
Culture medium—4 ml. of 10 per cent horse serum for each flask; horse serum is chosen because it is free of rinderpest antibody and generally available in most laboratories.
Incubation—five to seven days at 38 C.; the culture medium maintains a pH between 7.4 and 7.8.

PROPAGATION OF VIRUS AND DEVELOPMENT OF CF ANTIGEN

Relation Between EID and AgD.—Throughout the experiments, both virus and antigen titers were comparatively determined in 38 culture samples. The conditions common to these cultures were that most were seeded in relatively large amounts of virus and that they were all incubated for five days. Relationships between the two titers are presented (table 1).

TABLE 1—Relationships Between EID* and AgD†

	Number of samples with EID				
AgD	$\geq 10^{-3}$	10^{-4}	10^{-5}	$\leq 10^{-6}$	Total
<10	6	4	..	..	10
10	2	4	4	..	10
20	..	..	3	..	3
40	..	5	2	..	7
80	..	..	3	1	4
160	..	..	2	2	4
Total	8	13	14	3	38

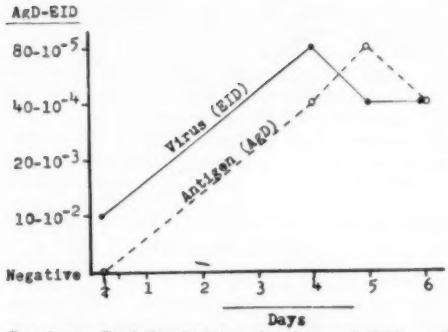
* EID = limit dilution infective to embryonating eggs; † AgD = limit dilution positive in CF antigen test.

The virus and antigen titers roughly correlated, on the whole. Groups showing higher titers for virus gave higher average titers and positive rates for antigen. All the 17 samples having virus titers of 10^{-5} or higher were positive in the CF test.

However, when the data are individually investigated, a number of cultures which were highly infective were negative in the CF test, or vice versa.

Periodical Determinations of EID and AgD.—Two series of tissue cultures were prepared under standard conditions. Both series were seeded with the same spleen virus, having a titer of EID 10^{-6}, but in different final dilutions, *i.e.*, 10^{-2} for one series and 10^{-5} for the other. Virus and antigen were then periodically and comparatively titrated in each of the series (graphs 2, 3).

In this experiment, the early stage of virus propagation was not observed. When tested at four days after incubation, the virus had reached its highest titer (EID



Graph 2—Periodical determinations of EID and AgD. Virus dilution seeded: 10^{-2}

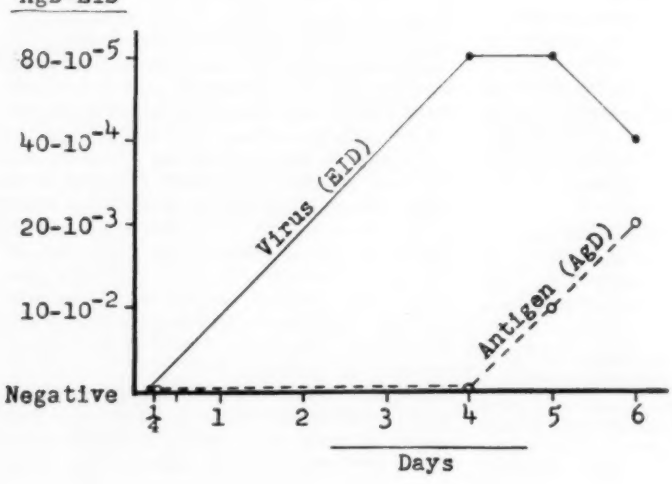
10^{-5}) irrespective of the amount of virus seeded. As compared with the propagation of virus, the development of antigen was delayed for some time. This was particularly remarkable in the culture started with a smaller amount of virus. While the five-day culture gave the highest antigen titer (AgD 1:80) in the first series, only an antigen of low titer (AgD 1:10) was first demonstrated on the same day of culture in the second series.

The relationships between titers of virus and antigen were often not uniform among samples obtained from different series at different periods. For instance, while the five-day culture of the first series had EID 10^{-4} and AgD 1:80, the four-day culture of the second series had EID 10^{-5} and was negative in the CF test at its highest concentration of 1:10. Similar results were obtained among the samples described (table 1). The discrepancies have been so evident both in frequency and in degree that they are hardly ascribed to certain technical errors. Thus it appears that the CF antigen and the infective virus may not be the same entity and, therefore, the antigen titer is not the index of the amount of infective virus.

CF Method as a Diagnostic Means in Tissue Cultures.—If the CF antigen is not the same entity as the infective virus, it must be a substance which is produced specifically as a result of virus propagation. This has been recognized not only in tissue cultures but also in such susceptible animals as cattle, rabbits, and chicken embryos. Based on this observation, the demonstration of CF antigen has been successfully applied to the diagnosis of rinderpest infection in these animals.

In the tissue culture experiments hitherto performed, 33 cultures were prepared under the standard conditions and incubated for five or more days before CF antigen was titrated in the cultured tissue. In these culture samples, relationships between the kind and dilution of the seed virus and AgD of the cultured tissue are presented (table 2).

The active limit of dilution was 10^{-5} for the spleen virus and 10^{-3} or 10^{-4} for the body virus. Although the titer of the seed virus was not estimated for each culture, it was presumed, from the results of titra-



Graph 3—Periodical determination of EID and AgD. Virus dilution seeded: 10^{-5}.

TABLE 2—Relationships Between Kind and Dilution of Seed Virus and AgD* of Cultured Tissue

	No. of samples seeded with								
	Spleen virus				Body virus				
AgD	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	Total
<10	....	....	....	....	....	..	....	6	6
10	....	....	....	1	....	....	....	1	2
20	....	....	....	....	....	....	....	....	....
40	....	....	....	....	....	....	12	....	12
80	1	3	....	....	....	....	6	....	10
160	....	1	....	....	1	1	....	....	3
Total	1	4	....	1	1	1	18	7	33

* AgD = limit dilution positive in CF antigen test.

tions hitherto performed, that the EID was approximately 10^{-6} for the spleen virus and 10^{-4} or 10^{-5} for the body virus. If this is correct, the limit dilution of seed virus which produces a demonstrable amount of CF antigen in the tissue culture is commonly EID 10.

Finally, two experiments were performed, in each of which virus was titrated, comparatively, in embryonating eggs and in tissue cultures (table 3). The spleen virus was serially diluted in Tyrode's solution with a factor of ten. For the titration in eggs, each virus dilution was inoculated intravenously into 13-day embryonating eggs, and results were determined by the CF test. This was the usual titration method for EID. For the titration in tissue cultures, serial groups of cultures were prepared by using the serial dilutions of virus for the culture mediums. The other conditions were standard, and culture was continued for five days. Then two methods were applied to the determination of the result in each culture group, *i.e.*, one to demonstrate the CF antigen directly in the cultured tissue and the other to transfer the cultured tissue into embryonating eggs which were finally judged by the CF test.

TABLE 3—Comparative Virus Titrations in Embryonating Eggs and in Tissue Cultures

	Virus titers	
Method of titration	Exper. 1	Exper. 2
In eggs, diagnosed by CF	10^{-3}	10^{-4}
In tissue cultures, diagnosed by CF	10^{-3}	10^{-3}
In tissue cultures, diagnosed by egg transfer	10^{-5}	10^{-5}

When determined directly by the demonstration of CF antigen, the virus titer measured in eggs was on the same level with that in tissue cultures in one experiment, and the former was one factor higher than the latter in the other experiment, *i.e.*, the tissue culture titer was the same or one factor lower than EID. When the tissue culture titer was determined indirectly by the transfer into eggs, it was, in both experiments, two factors higher than the directly determined tissue culture titer. Thus, compared with the ordinary egg-inoculation method, the tissue culture method in combination with the CF test for diagnosis appears to be somewhat less sensitive in detecting a minute amount of the virus.

VIRUS NEUTRALIZATION IN TISSUE CULTURES

Using the tissue culture method, neutralization tests of the LA virus by convalescent cattle and rabbit serums have been

TABLE 4—Virus-Neutralization Test in Tissue Culture

	CF* of tissue cultured with								
	Convalescent rabbit serum				Normal rabbit serum				
Virus seeded	A	B	C	D	E	F	G	H	Blank (control)
1:100	0	0	0	−3	....	....	....	....	....
1:300	0	0	−3	−3	....	....	....	....	....
1:900	0	0	−3	−3	100	95	100	100	100
1:2,700	....	....	....	....	100	89	68	30	38
1:8,100	....	....	....	....	22	0	5	5	0
1:24,300	....	....	....	....	−3	0	0	0	0

CF = complement-fixation titer; ≤ 10 = negative; 11 to 20 = doubtful; 21 to 100 = positive.

carried out with satisfactory results. One example is given (table 4).

Four normal and four convalescent (from the lapinized virus) rabbit serums were tested. For each serum, three or four test tubes were prepared in which serum, virus, and normal horse serum, each diluted with Tyrode's solution, were mixed together in the following proportions:

Serum to be tested, undiluted.....0.5 ml.
(or Tyrode's solution as control)
Normal horse serum, 1:7.....7.0 ml.
Virus diluted in a threefold series....7.5 ml.

The virus dilutions were from 1:50 to 1:450 for convalescent serums and from 1:450 to 1:12,150 for normal serums and control. In the final mixture, the dilutions of the virus were doubled; that of the serum to be tested was 1:30, and that of the whole serum element (serum to be tested plus normal horse serum) was 1:10. The mixtures were kept in ice water for one hour.

Cultures were prepared under standard conditions, except for the use of the mixtures instead of the ordinary culture medium, and incubated for seven days. The virus propagation in each group of cultures was then diagnostically examined by the CF test.

The virus was infective or, more exactly, "antigen-productive," up to a dilution of 1:2,700 (in one case, 1:8,100) in the presence of normal rabbit serum or in the blank, while it was inactive even in the highest concentration tested, *i.e.*, 1:100, in the presence of convalescent rabbit serum.

SUMMARY

The lapinized-avianized (LA) strain of rinderpest virus was successfully propagated in the culture of chicken embryo tissue. A simple suspension method similar to the Maitland method gave satisfactory results. Various culture conditions were studied for the establishment of a standard culture method.

As a result of virus propagation, specific complement-fixation (CF) antigen developed in the cultured tissue. Although this antigen appeared, in all probability, not to be the same entity as the infective virus, its demonstration provided a reliable means of diagnosis.

Compared with the embryonating egg-inoculation method, the tissue culture method, in combination with the CF test, appeared to be somewhat less sensitive in detecting a minute amount of virus. Nevertheless, the method was reliable for the determination of a relative titer of the virus.

This technique was also effectively applied to the virus-neutralization test.

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The Pathology of Experimentally Produced Histomoniasis in Turkeys

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THIS STUDY is concerned with the lesions produced by *Histomonas meleagridis* in the various organs of the turkey.

Characteristic lesions due to *H. meleagridis* are produced in the ceca and liver. One or both ceca may be involved, and liver lesions occur in practically all affected birds. Raised, thickened, yellowish ulcerations and occasional hemorrhagic areas appear on the serosa of the ceca. The ceca are enlarged and the lumina filled with a tough, yellowish core. The liver is enlarged and its surface is covered with small, round, yellow-green, depressed lesions.

Typical lesions have been reported by Curtice,[2] Pernot,[16] Smith,[21] Tyzzer,[23] Graybill,[6] Rettger and Kirkpatrick,[18] Doyle,[5] Picard,[17] Santos,[19] and McKay and Morehouse.[15] Shope[20] described "caseous" necrotic areas in the liver and ceca.

Farmer *et al.*[7] observed hyperemia, edema, and polymorphonuclear infiltration of the mucosa and submucosa of the ceca in the early stages of enterohepatitis. In later stages, the inflammatory reaction and protozoal infiltration extended into the muscular layers and the ceca exhibited "nests" of protozoa. They found that dense areas of "round cell" infiltration, cloudy swelling, degeneration, and necrosis of the cells characterized the liver lesions. Giant cells contained protozoa. They[7] described the lesion in the liver as, "a karyolytic necrosis accompanied by a subacute type of inflammatory response."

Chester and Robin[1] observed organisms within liver cells, but were not certain if these were Histomonas organisms. Desowitz[4] reported protozoan organisms in epithelial cells of the gut in turkeys dying from histomoniasis. Most authors are now in agreement with Tyzzer[22] and Smith[21] that the histomonads observed in lesions of the ceca and liver are intercellular.

Lesions produced by *H. meleagridis* have been reported in organs other than the cecum and liver. Chester and Robin[1] observed pale, mottled areas in the kidney, and necrosis of the spleen. Secondary lesions of the lungs and, rarely, of the kidneys were seen by Tyzzer and Fabyan.[23] Devolt and Davis[5] produced inoculation site and lung lesions by inserting liver infected with histomonads beneath the skin; a fatal sinusitis resulted from implantation of the infected tissue on the mucosa of the nasal sinus. Microscopic examination of round, white spots in a kidney showed an infiltration of mononuclear cells, areas of necrosis, and histomonads which appeared identical with those observed in the liver (Levine[12]). Kuprowski[10] reported congestion of the parabronchi of the lungs and enlargement and congestion of the spleen and kidneys. A small focus of protozoa was reported by Farmer *et al.*[7] in the spleens of turkeys which died 12 days after infection; no tissue reactions were observed. When McGuire[14] transfused cecal vein blood of a diseased bird into the wing veins of susceptible turkeys, lesions were produced in the lungs, kidneys, heart, and proventriculus.

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MATERIALS AND METHODS

Eight, 45-day-old Beltsville White turkeys were inoculated orally with embryonated *Heterakis gallinae* ova (300 to 600 ova/ml.). Seven of the inoculated birds died from histomoniasis and 1 recovered. Eight birds of the same age served as controls.

Necropsies on all of these turkeys either were done as soon as possible after death, or the carcasses were refrigerated until necropsy could be performed. The control birds and the 1 bird which recovered were exsanguinated. All organs were observed for gross lesions.

Bouin's-fixed specimens of ceca, livers, hearts, and spleens were dehydrated and infiltrated according to the butyl alcohol-paraffin mush method of Johnston *et al.*[9] Sections were cut and stained with Harris' hematoxylin and eosin stain, as modified by Malewitz and Smith.[13] Periodic acid Schiff (PAS) reaction was used on all specimens (MacManus[11]).

RESULTS AND DISCUSSION

The first signs of infection, which appeared between nine and 21 days after inoculation, were sulfur-colored feces, followed by drowsiness, drooping of the wings, and anorexia. Infected turkeys began losing weight 12 days after inoculation. The average rectal temperature of the control birds was 106.6 F. The temperatures of the infected turkeys rose, on the average, to 108.2 F. between 15 and 21 days after inoculation, then decreased

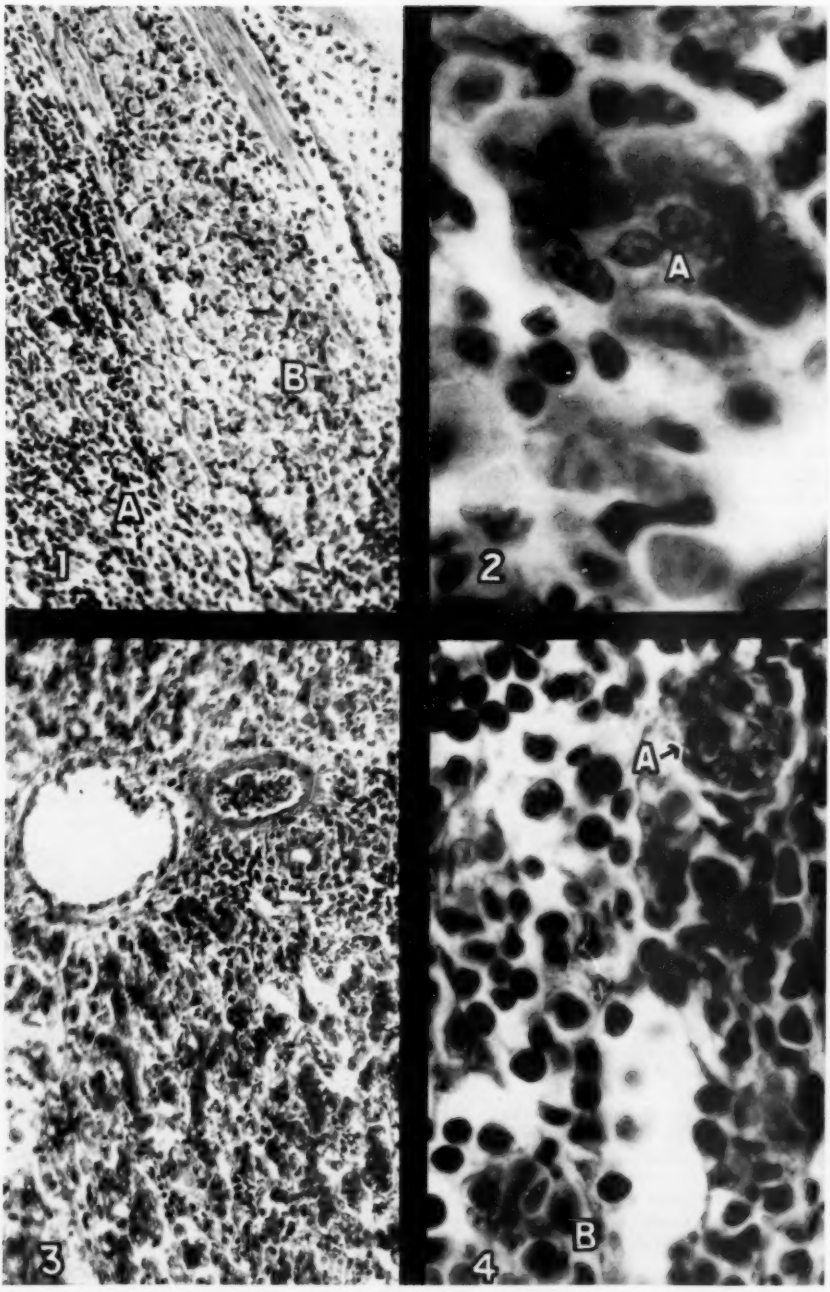

Fig. 1—Necrosis of cecum of turkey with inflammatory reaction (A) and nests of protozoa (B). H & E; x 125.

Fig. 2—Multinucleated giant cell (A) with protozoa in cecum. H & E; x 1,650.

Fig. 3—Coagulation necrosis of turkey liver. H & E; x 230.

Fig. 4—Multinucleated giant cell (A) and protozoa nests (B) in liver. H & E; x 1,800.

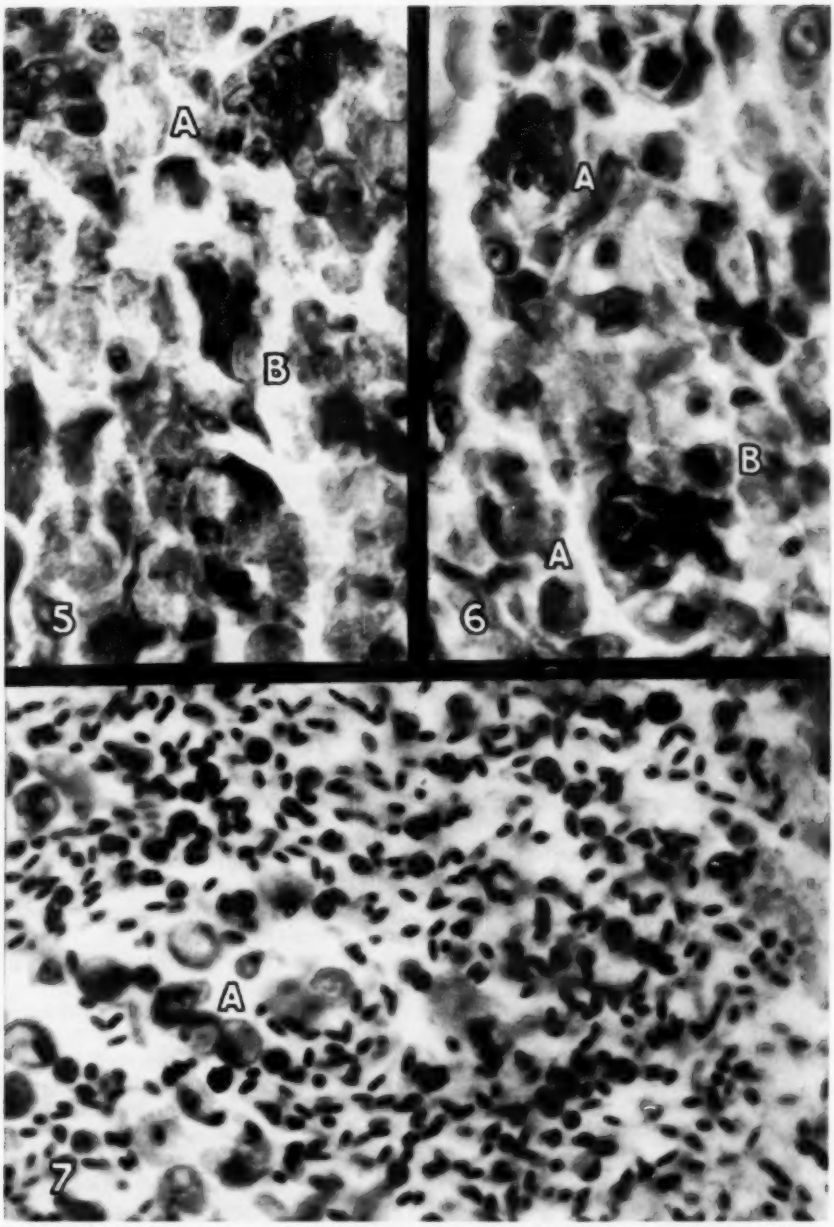

Fig. 5—Multinucleated giant cell (A) and protozoa nests (B) in kidney of a turkey. H & E; x 1,500.

Fig. 6—Multinucleated giant cell (A) and protozoa nests (B) in turkey spleen. H & E; x 1,800.

Fig. 7—Protozoa (A) in blood vessel of the spleen. H & E; x 215.

to an average of 103.7 F. on the day of death.

Gross lesions were observed in the livers, ceca, kidneys, lungs, hearts, and spleens. The lesions in the spleen and kidneys appeared as small nodules on the surface and, in the lungs, as hemorrhagic areas. Hearts from infected turkeys were firm, with red clots in the atria. The ceca were enlarged and the wall appeared thin, with hemorrhagic areas in the serosa. The lumina of infected ceca contained cores of dry, yellow, caseous material. The liver was enlarged, with scattered, depressed, greenish-yellow, necrotic areas.

The microscopic lesions observed in the ceca were similar to those reported by Farmer *et al.*[7] Hyperemia and thrombi were present in the blood vessels. A diffuse type of lymphocytic and large mononuclear infiltration occurred in the mucous membrane and submucosa. This was a granulomatous, inflammatory reaction. The muscularis externa exhibited a focal lymphocytic infiltration in some areas, and edema was present in areas with little lymphocytic infiltration. There was coagulation necrosis (fig. 1), with histomonads penetrating all layers of the cecum, singly or in nests. Multinucleated, giant cells were numerous and often contained the protozoa (fig. 2). The smooth muscle of the muscularis externa appeared, in places, to be undergoing hyalinization.

The parasites observed in the ceca were identical with those previously described. The protozoa was ovoid in shape, stained light pink with hematoxylin and eosin, and appeared to be surrounded by a clear space. The nucleus was often invisible but, when observed, appeared to have a halo around it. Ovoid bodies, similar to those just described, stained red with the periodic acid Schiff (PAS) reagent as modified by MacManus.[12] It is suggested that these PAS-positive bodies may be one of the stages in the life cycle of *H. meleagridis*. Del Giudice[3] reported these PAS-positive bodies in the ceca and livers of fowl infected with *H. meleagridis*, and suggested that they were degenerating cells.

The blood vessels and sinusoids of the liver were congested. Serofibrinous exudate was present in areas of coagulation necrosis which occurred in scattered areas in the liver parenchyma. Whether these areas were centrally or peripherally located in the liver lobules could not be determined. The necrotic areas were infiltrated with lymphocytes, macrophages, and numerous giant cells (fig. 3). As in the ceca, this was a granulomatous, inflammatory reaction. The parasites appeared in nests, frequently in the sinusoids, and often within multinucleated giant cells (fig. 4). Liver sections stained with PAS reagent revealed numerous ovoid bodies which stained red, as observed in the ceca.

The lesions in the kidney were similar to those observed in the ceca and liver. Congestion and hemorrhage were apparent in circumscribed areas. Lymphocytes infiltrated into the areas of caseation necrosis. Multinucleated giant cells and parasites were observed in the necrotic areas (fig. 5).

The spleen had lesions similar to those of other organs. Nests of protozoa, surrounded by macrophages and lymphocytes, were observed (fig. 6). The macrophages were distributed more diffusely throughout the spleen than in other organs. The inflammatory reaction was purely granulomatous, with an almost complete absence of multinucleated giant cells. Protozoa were numerous in the blood vessels (fig. 7).

There was congestion and hemorrhage in the lung, particularly in the air sacs. Peribronchial edema was apparent, and contained numerous heterophils and macrophages. Focal lymphocytic infiltration was observed. Histomonads could not be demonstrated in the lung.

Congestion was observed in the blood vessels of the pancreas. The mesentery was inflamed and edematous with a serocellular exudate, but necrosis was absent, and parasites could not be demonstrated in the sections examined.

An epicarditis, characterized by edema and a serocellular exudate, was prominent. The epicardium was greatly thickened in places.

Histomonads were observed in the ceca, liver, kidney, and spleen. The lesions in the heart, pancreas, and lungs probably were secondary; parasites could not be demonstrated in these organs.

There were no signs of infection and no gross or microscopic lesions in the control birds when they were killed, 30 days after the last infected bird died.

SUMMARY

Typical gross lesions were observed in the ceca, livers, spleens, kidneys, and lungs

of turkeys experimentally infected with *Histomonas meleagridis*.

Microscopic lesions, in which the parasite could be demonstrated, were observed in the ceca, livers, kidneys, and spleens. The lesions were characterized by hyperemia, hemorrhage, lymphocytic infiltration, macrophages, the presence of multinucleated giant cells, necrosis and, usually, a serous exudate. This was a granulomatous type of inflammation, accompanied by necrosis.

In the lungs, pancreas, and heart, there were areas of hyperemia and serocellular exudate, but parasites were not demonstrated. Lymphocytic infiltration was observed in the lungs.

The control birds remained healthy, and no gross or microscopic lesions were found at necropsy.

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Lesions in the Cerebellum and in the Reticular and Vestibular Centers in Newcastle Disease

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NEWCASTLE DISEASE is a contagious disease of poultry caused by a specific virus which has both neurotropic and pneumotropic properties. While variations have been demonstrated in the neurotropism of different strains,[1, 12] that of California strain 11914 has been well established. Studies have shown that this strain of the virus was definitely neurotropic on intramuscular injection in the chicken,[13] and that this characteristic remained constant even after adaptation to mammals.[7] It has been assigned one of the highest neurotropic indexes of any of the strains studied.[1, 12]

Suddenly developing tremors, affecting one or more of the extremities or the head and neck, are the clinical manifestations of this neurotropism.[13] Lesions in the nerve centers concerned with postural regulation would be expected in chickens exhibiting such signs. The most important centers governing postural regulation in the chicken are the cerebellum and the reticular and vestibular nuclei.

This experiment was undertaken in an attempt to determine whether lesions in any of these centers were produced by Newcastle disease, and to determine the type, specific location, constancy, and severity of such lesions. Histological examinations were limited to these centers because preliminary work had indicated that they offered the most fertile field for investigation.

REVIEW OF LITERATURE

Newcastle disease was first reported in 1926 by Kraneveld[16] and by Doyle.[9] It first appeared in the United States in 1940, although it was not identified until 1944. The history of Newcastle disease from its first occurrence in the United States until it was identified by Brandly *et al.*[6] is contained in the reports of Beach[2, 4, 5] and of Stover.[21] In addition to these reports, a voluminous literature has accumulated, but only a few of these papers are concerned with the changes occurring in the central nervous system.

As early as 1932, Fukushima *et al.*[11] and Fukushima and Shimomoru[10] studied the changes produced by this disease. They found regressive changes of the neurons, proliferation of glial cells and perivascular lymphocytic infiltrations. They also observed the occasional occurrence of heterophils in the lesions and, in two cases, large necrotic lesions. These changes were limited to the medulla oblongata and the cerebellum.

Jungherr and Minard[14] and, later, Jungherr *et al.*[13] reported capillary hemorrhage, endotheliosis, neuronal changes, and foci of glial proliferation in the spinal cord, medulla oblongata, cerebellum, and thalamus.

Karzon and Bang[15] reported primary neuronal injury, inflammatory infiltration of the tissues, and perivascular lymphocytic infiltration to be generally disseminated throughout the brain following intramuscular injection of the GC 179 strain of Newcastle disease virus.

Auer[3] described extensive hyperemia, lymphocytic infiltration and some degeneration of ganglion cells in field cases of Newcastle disease.

MATERIALS AND METHODS

Healthy, 5-week-old White Plymouth Rock chickens were allotted to two groups of 50 birds each at the beginning of this experiment. The groups were housed in separate buildings, and were fed a starter mash * which did not contain an antibiotic.

A pooled sample, composed of equal amounts of serum taken from 5 birds, was examined serologically at the time the chickens were grouped. The hemagglutination-inhibition tests for Newcastle disease were negative at this time, as were the serum-neutralization tests for infectious bronchitis and the plate-agglutination tests for chronic respiratory disease.

The virus used in this experiment was the California strain 11914 which had undergone approximately 15 passages in eggs.

Each of the chickens in the experimental group received 0.1 ml. of the inoculum intramuscularly in the right thigh. Individual birds were killed when they developed clinical signs of Newcastle disease. To minimize postmortem autolysis, tissues were selected from chickens which could be killed or which were known to have been dead for not more than 20 minutes. The chickens were

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* Prepared by the Division of Poultry Husbandry, University of Illinois, Urbana.

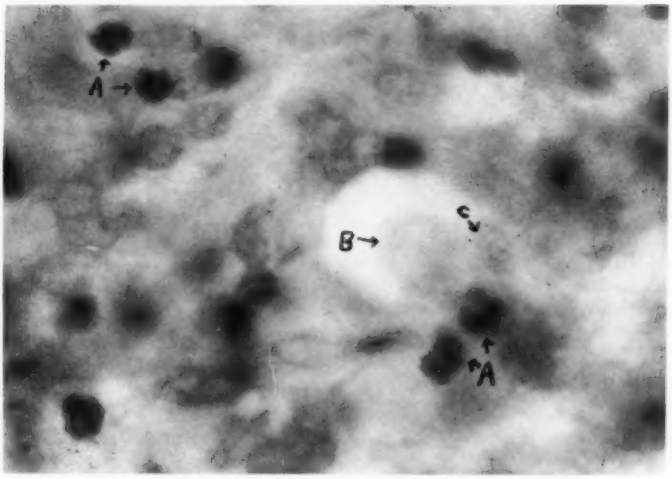

Fig. 1—Medulla oblongata of chicken 4178, showing an acute lesion in the superior vestibular nucleus with many heterophils (A), and one neuron which is undergoing necrobiotic changes (B). There is some early proliferation of glial cells (C). Azure eosin stain; x 1018.

killed by crushing the ninth or tenth cervical vertebra by applying an emasculatome.

The brain and the first segment of the cervical spinal cord were removed from each of the exposed chickens within 20 minutes after death. The tissues were frozen for later virological examination or were fixed in formalin. After a preliminary fixation of six to eight hours, the brains were divided into segments of 3 to 5 mm. in thickness, which were placed in formalin for at least 48 hours to complete fixation. After completion of fixation, the tissues were processed and embedded in paraffin.

This method of fixation was chosen for a number of reasons. Chromatin was not considered desirable because it would preclude the use of silver impregnation methods. Perfusion of the brain *in situ* was excluded because preliminary work had shown that even the slight amount of pressure necessary to insure penetration of the fixative to all areas of the brain would produce some distention of the blood vessels.

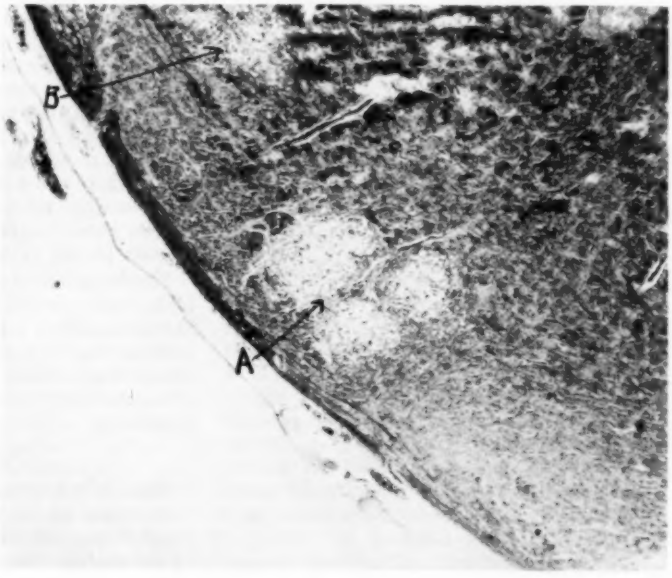

Fig. 2—Medulla oblongata of chicken 4168, showing two large well-defined foci of liquefaction necrosis. One is in the superior olive (A), the other in the arcuate fibers of the nucleus laminaris (B). Weil stain; x 37.5.

Serial sections, 10μ in thickness, were prepared from the brains of 13 of the exposed and 5 of the unexposed birds. The areas selected for study extended from the rostral end of the nucleus tecti, caudally to the level of the nucleus laminaris. This area includes all of the cerebellar and vestibular nuclei and the medial reticular nucleus. At intervals of 30 sections, two or three consecutive sections were selected and mounted on slides. The unused sections were stored for future use. The mounted sections were stained by the azure eosin method of Wintrobe,[22] by a modification of the Weil technique of Conn and Darrow [8] and, in a few instances, by the silver method of Romanes.[19]

The brains of 4 of the exposed chickens were examined virologically, and Newcastle disease virus was recovered.

RESULTS

Clinical signs of Newcastle disease were apparent in the experimentally inoculated chickens by the forty-fifth hour after injection. These signs were: drowsiness and somnolence, deficiencies in the nervous control of muscular activity and, terminally, paralysis and coma. Inability to maintain the posture of the head, and tremors were seen as muscular deficiencies. The tremors were rhythmic clonic contractions of various muscle groups, at a rate of 80 to 228 per minute. The involved muscles were usually those of the legs and neck, although the wings were occasionally affected. There was no evidence of decerebrate rigidity in these birds. After the development of tremors, the chickens were incapable of maintaining their normal posture. These changes suggested the probability of derangement of the proprioceptive reflex mechanisms.

The unexposed control birds remained free of disease throughout the experiment. The hemagglutination-inhibition test and the serological tests for infectious bronchitis and chronic respiratory disease remained negative. All of the exposed chickens had died of Newcastle disease, or had been killed, by the ninety-sixth hour after exposure.

Microscopic studies included the brains of 13 of the exposed birds and 5 of the unexposed controls. The exposed chickens were killed at various times after inoculation. Lesions were found in the vestibular nuclei of all the exposed chickens, and in the medial reticular nuclei of all but 3. Lesions in the cerebellar nuclei were found in all birds which survived for more than 48 hours.

The most constant and striking change observed in the infected chickens was an extreme congestion of the entire vascular bed of the medulla oblongata and of the cerebellum. This was accompanied by proliferation of the endothelial cells, indicated by the plumpness of the cells in contrast to their normally thin, elongated appearance. Neuronal degeneration was present in the brains of all the affected chickens. These changes varied from beginning chromatolysis to frank necrosis. Affected neurons had undergone vacuolation, hyalinization or phagocytosis, or had simply disappeared, with resultant cavitation of the tissues. They were found uniformly in all of the vestibular nuclei and, usually, in the reticular and cerebellar centers. Identical changes were found in the Purkinje cells, but there was no uniformity in distribution.

Frankly necrotic changes involving the neurons, fiber tracts, and stroma were seen in six of the eight brains which were selected approximately 48 hours after injection. The lesions varied greatly in size and severity, some being small and acute and containing many heterophils. In these, the neurons were usually the only tissue elements destroyed (fig. 1). Other lesions consisted of large, well-defined areas of liquefaction necrosis, with only a reduced amount of stroma and a few heterophils remaining (fig. 2, 3). Both acute lesions and foci of necrosis were seen in the brains of chickens which were killed 48 hours after infection. Replacement gliosis, which probably represented an attempt at repair, was found in corresponding locations in the brain of 1 bird which survived for 96 hours. Thus, the changes appeared to have occurred in a definite sequence. Acute inflammation was followed by focal necrosis and, finally, by replacement gliosis. The lesions were generally restricted to the vestibular nuclei or to the superior olive.

The brains of 5 of the unexposed control birds were processed in the same way as the tissues from infected birds. All of these sections were essentially normal. Some chromatolysis was seen but, in the absence of more drastic changes, was not considered significant.

DISCUSSION

The outstanding lesion was pronounced hyperemia of the medulla oblongata and cerebellum, accompanied by proliferation of the endothelial cells, and was uniformly

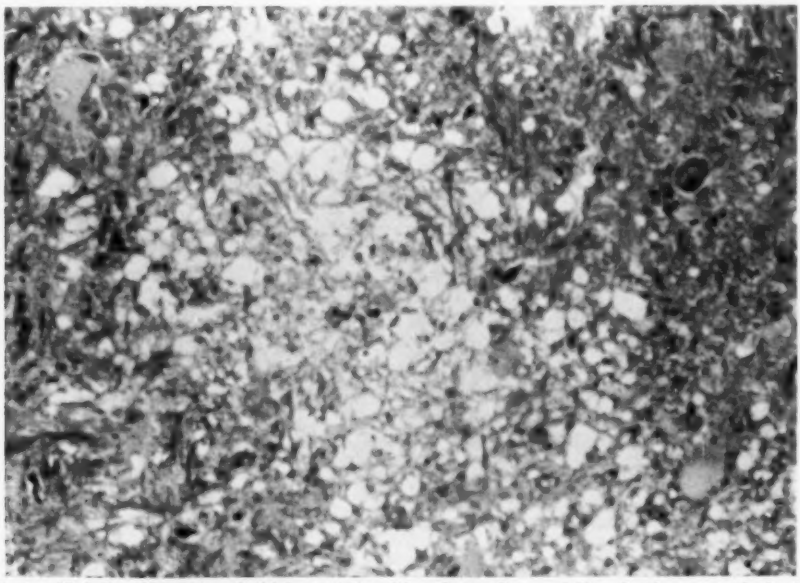

Fig 3—Medulla oblongata of chicken 4171, with a large focus of liquefaction necrosis in the dorsolateral, vestibular nucleus. Weil stain; x 160.

encountered. Vascular engorgement, occurring in an organ as rigidly confined as the brain, would result in increased pressure within the tissue, which could account for the appearance of drowsiness and somnolence as clinical signs of Newcastle disease.

Neuronal degeneration was found in the brains of all the infected chickens, mainly affecting structures which are important to the completion of proprioceptive reflexes. Sherrington [20] has called the cerebellum an organ of confluence for afferent proprioceptive impulses from the muscles and from the labyrinths. Impulses arising in the muscles would reach the cerebellar cortex of the chicken via the spinocerebellar tracts. They would then pass via the fibers of Purkinje cells to the cerebellar nuclei. Most afferent impulses of labyrinthine origin would pass through the vestibular nuclei, although some may reach the cerebellar nuclei by way of direct vestibular fibers. The system formed by the fibers of the Purkinje cells is one of the most important networks in the cerebellum of the chickens. It reinforces proprioceptive impulses and channels them through specific nuclei. The vestibular nuclei are also extremely important in proprioceptive reflexes and are closely related to the reticular nuclei which have similar functions. Kappers *et al.*[15] mention the close evolutionary relationship of these two groups of nuclei. Kemberling *et al.*[17] have shown that the reticular nuclei can compensate for injury to the vestibular nuclei and vice versa.

The total effect of the changes which were found would be a gross distortion of the proprioceptive responses of the chicken. Efferent activity in structures activated or inhibited by the damaged nuclei would result in an alteration of the pattern of facilitation or inhibition at the level of the spinal cord. Such an alteration could be responsible for the tremors and the inability to maintain the posture of the head which were seen as clinical signs of Newcastle disease in these birds.

SUMMARY

Newcastle disease was induced in susceptible, 5-week-old chickens by the intramuscular injection of California strain 11914 of Newcastle disease virus. All inoculated chickens died of the disease or were killed within 96 hours. The signs of Newcastle disease in these birds were drowsiness, tremors, inability to maintain the posture of the head and, finally, paralysis and coma. The birds were unable to

maintain their normal attitudes after the appearance of the tremors.

The predominant tissue change was an extensive hyperemia of the central nervous system, accompanied by proliferation of the endothelial cells.

Degenerative changes of the neurons, ranging from mild chromatolysis to frank necrosis, were found in all cases, primarily in the vestibular, reticular, and cerebellar nuclei, and in the Purkinje cells.

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Factors Influencing the Growth of Newcastle Disease Virus in Isolated Chorioallantoic Membranes

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NEWCASTLE DISEASE VIRUS grows readily in isolated chorioallantoic membranes suspended in Tyrode's solution and incubated at 37 C., as shown by Scott *et al.* in 1953.[13] As this simple system is easily altered to favor or suppress virus growth, a systematic study of the requirements of Newcastle disease virus for optimal growth was undertaken.

MATERIALS AND METHODS

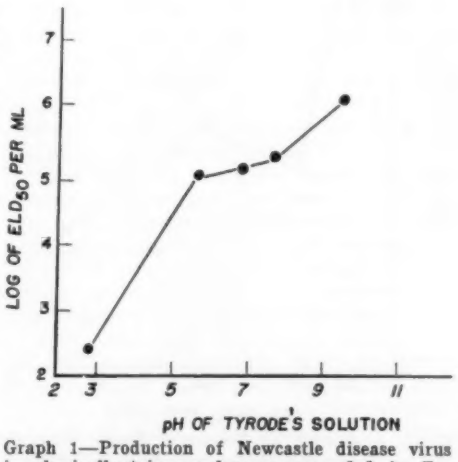
The Roakin-NJ-1946 strain of Newcastle disease virus (NDV) was obtained from the interregional repository for NDV strains, Department of Veterinary Science, University of Wisconsin. Fertile eggs, from apparently healthy, white Leghorn chickens free of pullorum disease, Newcastle disease, and infectious bronchitis, were used for tissues in culturing and in the titration of the virus.

The concentration of virus was determined by titrating its lethal activity for embryonating chicken eggs and its agglutinative activity for chicken erythrocytes. Tenfold serial dilutions of virus were made in sterile nutrient broth. Five embryonating eggs, 10 or 11 days old, were inoculated per dilution, 0.1 ml. of the diluted virus suspension being injected into the allantoic chamber of each egg. Deaths of embryos from virus infection occurred between two and six days after inoculation. Specificity of embryonic death was determined by testing for the presence of hemagglutinins. Bacterial sterility of the cultures was determined in thioglycollate broth and on nutrient agar. Hemagglutination titrations followed the method of Salk.[12] The 50 per cent embryo lethal dose ($e.l.d._{50}$) was calculated according to the method suggested by Reed and Muench,[11] on the basis of accumulative deaths or survivals of embryonating eggs.

The method of culturing virus in isolated, respiring chorioallantoic membranes suspended in Tyrode's solution has been described by Scott *et al.*[13] and is a modification of the procedure used by Fulton and Armitage.[4] Essentially, the method involves the aseptic removal of the chorioallantoic membrane (c.a.m.) from 10- or 11-day-old embryonating chicken eggs and washing the membrane in three bottles, each containing 200 ml. of saline solution. The washed c.a.m. was then suspended in 19 ml. of Tyrode's solution, to which 1 ml. of NDV, containing approximately $10^{2.3}$ $e.l.d._{50}$ per milliliter, was added. The culture was incubated at 37 C. Only the extracellular virus concentration in the culture fluids was determined in these studies, since the amount of bound (intercellular) virus was not appreciably different from that of liberated virus at any given time during the growth of NDV. The amount of virus in the culture fluids was usually determined after 24 hours at 37 C.

Tyrode's solution was used as the suspending medium for the respiring c.a.m. cultures, also referred to as tissue cultures. The ingredients of this solution were made up separately in tenfold concentrations and sterilized by autoclaving, except the sodium bicarbonate solution, which was sterilized by filtration. The method of preparation permitted change in the concentration of any of the ingredients of Tyrode's solution.

The hydrogen ion concentration of Tyrode's solution was varied by the addition of 1 N HCl or 1 N NaOH prior to the addition of the c.a.m. or virus. The pH of Tyrode's solution did not change appreciably after addition of virus or of c.a.m. One milliliter of a 10^{-6} dilution in saline solution of NDV was added to each culture.



Graph 1—Production of Newcastle disease virus in chorioallantoic membranes suspended in Tyrode's solutions of different pH values.

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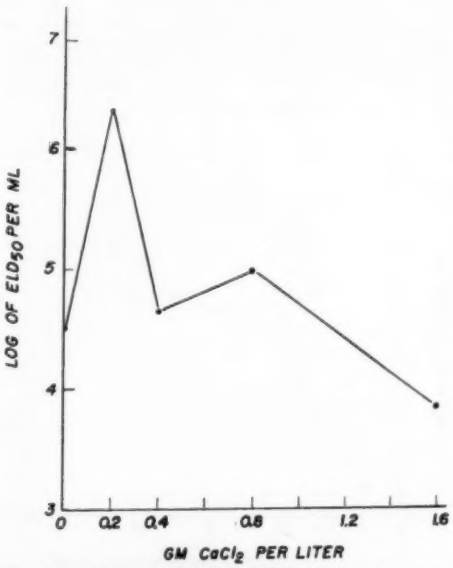
RESULTS

The hydrogen ion concentration of the suspending medium had a decided effect on the production of virus in isolated chorioallantoic membranes (graph 1). Highest titers were obtained in cultures with an initial pH of 9.5. Since, according to Cameron,[3] the optimum pH for culture of chicken tissue is 7.6, it appears that optimum pH for virus growth differs from that of the cell. Perhaps the pH influences the solubility of some of the cations in Tyrode's solution or the function of some vital enzyme system, e.g., an alkaline phosphatase. Although the titer of virus obtained at a pH of 2.7 was low ($10^{2.4}$ e.l.d.$_{50}$/ml.) it was assumed that the virus must have grown, since the initial inoculum ($10^{2.3}$ e.l.d.$_{50}$/ml.) would not be expected to persist unchanged for 24 hours at 37 C. Moses and co-workers [8] found that the maximum stability of four strains of NDV was between pH 4 and pH 10 at 37 C. after 24 hours. They did not find an increase in virus titer at any pH level after 24 hours at 37 C. It was, therefore, assumed in this work that the pH influenced the production of virus from the c.a.m. and was not a factor affecting viral aggregation.



Graph 2—Growth of Newcastle disease virus in chorioallantoic membranes suspended in Tyrode's solutions containing different concentrations of $CaCl_2$ and incubated at 37 C. for 24 hours.

The pH of the Tyrode's solution was not determined after the growth of NDV in these studies. Later work [14] indicated that infection of the c.a.m. with NDV retarded any appreciable change in the pH of Tyrode's solution.

Newcastle disease virus grew best when $cacl_2$ was at a concentration of 0.2 Gm. per liter of Tyrode's solution (graph 2). Titer of the virus was determined after incubation of the culture at 37 C. for 24 hours with a given concentration of $cacl_2$. Virus growth decreased as the $cacl_2$ concentration was increased from the 0.2 Gm. per liter level, except for a slight increase at the 0.8 Gm. per liter level (graph 2).

TABLE 1—Titers of Newcastle Disease Virus Adsorbed by Chorioallantoic Membranes Suspended in Tyrode's Solution Containing Various Concentrations of $CaCl_2$*

Concentrated $CaCl_2$ (Gm./liter)	e.l.d $_{50}$/ml. at 37 C. after 1 hr.
Control (no c.a.m.)	$10^{1.9}$
0.0	$10^{3.9}$
0.2	$10^{2.3}$
0.4	$10^{2.8}$
0.8	$10^{2.5}$
1.6	$10^{2.5}$
3.2	$10^{2.5}$

* Incubation was at 37 C. for one hour.

The adsorption of the virus was also studied by suspending chorioallantoic membranes in Tyrode's solutions containing different concentrations of $cacl_2$, but with the same concentration of NDV. After incubation at 37 C. for one hour, the c.a.m. was removed and the virus titer in the Tyrode's solution was determined. Calcium ions did not seem to affect the adsorption of NDV on chorioallantoic membranes (table 1).

Calcium ions and other cations are reputed to be necessary for adsorption of the virus to the cells of its prospective host. Puck *et al.*[9] have shown higher levels of calcium increase adsorption and growth of the phage in some bacteriophage systems. However, the reverse occurred here. Increasing the concentration of $cacl_2$ tended to produce a decrease in the titer of NDV. The pH of Tyrode's solution was not implicated in producing this effect. It is possible that the calcium ions compete with other ions, such as potassium ions, which are required for virus reproduction. The mechanism by which $cacl_2$ depressed virus production, when present in concentrations above that normally found in Tyrode's solution, is not known at present.

TABLE 2—Titers of Newcastle Disease Virus Adsorbed by Chorioallantoic Membranes Suspended in Tyrode's Solution Containing Various Concentrations of KCl*

Concentrated KCl (Gm./liter)	e.l.d. $_{50}$/ml. at 37 C. after 1 hr.
0.0	$10^{3.2}$
0.2	$10^{2.8}$
0.3	$10^{3.2}$
0.4	$10^{2.8}$
0.6	$10^{2.8}$

* Incubation was at 37 C. for one hour. Initial virus titer of the medium was $10^{3.16}$ e.l.d.$_{50}$ per milliliter.

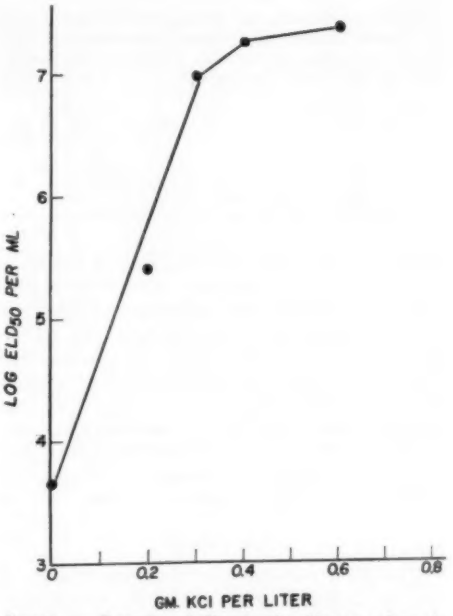
The titer of NDV in choriollantoic membranes suspended in Tyrode's solution increased with an increase in KCl in the solution (graph 3). The maximal virus titer was obtained in those solutions that contained 0.4 Gm. KCl per liter. Potassium apparently is required for the multiplication of NDV, as growth was minimal in Tyrode's solution from which KCl had been omitted. An experiment to determine whether potassium was required for adsorption of NDV by the c.a.m. did not reveal an adsorption pattern that differed significantly in low and high concentrations of potassium (table 2). Levine *et al.*[7] have also shown that the attachment of influenza virus to c.a.m. was not influenced by K^+. However, potassium was necessary for the growth of the virus in this tissue.

Addition of potassium ions to a potassium-deficient medium did enable the virus to grow. Magnesium ions could not be substituted for potassium. Cultures which contained, in addition to chorioallantoic membranes and virus, either 0.4 mg. KCl per milliliter of Tyrode's solution or 0.4 mg. $MgCl_2$ were incubated at 37 C. for 24 hours. Two other cultures deficient in KCl were incubated for 24 hours; KCl was added to one to obtain a concentration of 0.4 mg. per milliliter of solution, and the two cultures were incubated for an additional 24 hours. The NDV grew poorly in chorioallantoic membranes suspended in a potassium-deficient medium. Even after 24 hours of incubation, virus growth was resumed at an increased rate when potassium was added (table 3).

TABLE 3—The Influence of K and Mg Ions in Tyrode's Solution on the Growth of Newcastle Disease Virus in Chorioallantoic Membranes*

Conc. (mg./ml.) in Tyrode's		Total incubation time	
KCl	$MgCl_2$	(hr.) at 37 C.	e.l.d.$_{50}$/ml.
0.4	0.2	24	$10^{5.6}$
0.0	0.6	24	$10^{3.2}$
0.0	0.2	48	$10^{3.4}$
0.0†	0.2	48	$10^{5.3}$

* Initial virus titer was $10^{1.2}$ e.l.d.$_{50}$ per milliliter.
† 0.4 added after first 24 hours.



Graph 3—Growth of Newcastle disease virus in chorioallantoic membranes suspended in Tyrode's solutions that contained various concentrations of KCl and incubated at 37 C. for 24 hours.

When the concentration of $MgCl_2$ in the Tyrode's solution was altered, as was done with $CaCl_2$ and KCl, maximal growth occurred in Tyrode's solution which contained 0.1 Gm. of $MgCl_2$ per liter, the concentration normally used in Tyrode's solution. Concentrations of $MgCl_2$ above the 0.2 Gm. per liter value were detrimental to the production of NDV in chorioallantoic membranes (table 4).

The effect on virus production of different concentrations of glucose was studied in chorioallantoic membranes suspended in Tyrode's solution. The NDV was added to the respective c.a.m. cultures and the virus titer was determined after incubation for 24 hours. The experiment was done in triplicate. Although the results were not entirely consistent within each experiment, increases in the glucose concentration in Tyrode's solution produced corresponding decreases in the growth of NDV. An average of the results from the three experiments is presented (graph 4). In all instances, the

TABLE 4—Growth of Newcastle Disease Virus in Chorioallantoic Membranes Suspended in Tyrode's Solution Containing Different Concentrations of $MgCl_2$ and Incubated at 37 C. for 24 hours

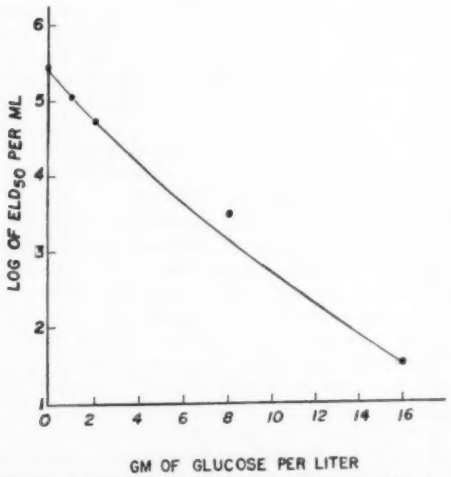
Conc. of $MgCl_2$ (Gm./liter)	e.l.d.$_{50}$/ml. at 37 C. after 24 hr.
0.0	0
0.2	$10^{6.5}$
0.4	$10^{5.1}$
0.6	$10^{3.0}$

highest virus titer was obtained in the solutions deficient in glucose. Addition of glucose to respiring chorioallantoic membranes infected with NDV was expected to stimulate the production of virus, as glucose was the only exogenous energy-supplying substrate in the culture, and the virus-host complex must have energy for the synthesis of virus. The results indicated that extracellular glucose is not necessary for the production of virus in c.a.m. cultures.

DISCUSSION

The observations that maximal yields of NDV in isolated chorioallantoic membranes were obtained at pH 9.5, and that potassium ion was required for production of virus may fit a pattern in virus synthesis.

The fact that maximal yields of NDV were obtained in tissues suspended in a medium at a pH of 9.5, and the finding that some alkaline phosphatases have a pH optimum around 9.5, suggests one relationship.



Graph 4—Growth of Newcastle disease virus in chorioallantoic membranes suspended in Tyrode's solutions containing different concentrations of glucose and incubated at 37 C. for 24 hours.

Hoaglund *et al.*,[5] in examination of the biochemical activities of purified suspensions of vaccinia elementary bodies, demonstrated phosphatases but not dehydrogenases. These authors felt that the enzyme was adsorbed to the virus particles but suggested that it was a functional characteristic of vaccinia virus. Beard[1] found that a highly purified and concentrated preparation of the virus of avian erythromyeloblastic leukosis contained an adenosinetriphosphate (ATP) transphosphorylase. This enzyme, which required potassium, could not be separated from the virus, as shown by electrophoretic and optical ultracentrifugation data. Beard discussed whether the enzyme was a specific part of the virus or adsorbed to the particles, as well as the possible role of the enzyme in virus growth. Racker and Kabat[10] investigated the changes produced by Lansing poliomyelitis virus in the brains of mice and found that ATP activity was increased in infected brains.

Potassium is required for the growth of other viruses. Levine *et al.*[6] found that influenza virus multiplication was inhibited in chorioallantoic membranes suspended in Hank's solution deficient in potassium, but that optimum virus growth was obtained in solutions that contained 0.4 mg. of KCl per milliliter. The same concentration of potassium permitted optimum growth of NDV. Boyer *et al.*[2] found potassium to be required for an ATP phosphopyruvic transphosphorylase in animal cells. Calcium ions depressed the enzyme. Potassium might function in a transphosphorylase in the c.a.m.

Glucose, the only energy-supplying compound in the Tyrode's solution, had an adverse effect on NDV growth under aerobic conditions. The phosphorylation of glucose requires ATP, as does the synthesis of virus; thus, the competition might inhibit the production of virus. This inhibition might occur only in the early stages of virus synthesis, since the measurements for virus were made when the culture had incubated only 24 hours. If there is a pool of high energy compounds in the c.a.m. which may be used for virus synthesis, the pool might be depleted after 24 hours; then glucose might be required for the production of energy. This possibility was not tested, but glucose was required in anaerobic c.a.m. cultures to produce high yields of NDV.[14]

Increases in the glucose concentration might cause the c.a.m. to produce acids

which could alter the pH of the Tyrode's solution, thereby influencing the virus growth. This explanation was not tested in these studies.

SUMMARY

Growth of Newcastle disease virus in tissue culture was affected by changes in hydrogen ion concentration and in the concentration of K, Ca, and Mg ions.

The maximal titer of Newcastle disease virus (Roakin strain) was obtained in cultures of chorioallantoic membranes suspended in Tyrode's solution at pH 9.5. The smallest yield of virus was obtained at pH 2.7. High yields of virus were obtained when the pH range of Tyrode's solution was 6.7 to 7.6.

The maximal titer of Newcastle disease virus was obtained from growth in chorioallantoic membranes incubated in Tyrode's solution that contained 0.2 Gm. of $CaCl_2$ per liter. Greater concentrations of $CaCl_2$ appeared to deter growth of virus in tissue culture.

Potassium was found to be essential for growth of Newcastle disease virus in tissue cultures. Maximum yields of virus were obtained in membranes suspended in Tyrode's solution that contained 0.4 Gm. of KCl per liter. Virus growth was inhibited in membranes incubated in Tyrode's solutions that were deficient in potassium. This inhibiting effect could be overcome by the addition of potassium to the solution. Magnesium ions did not overcome the inhibition of growth of virus in tissues suspended in Tyrode's solution deficient in potassium. Magnesium ions were detrimental to the production of virus in tissue culture when the magnesium exceeded the concentration normally found in Tyrode's solution.

Increasing the concentration of glucose in Tyrode's solution produced an inhibitory effect upon the growth of virus in tissues suspended in these solutions.

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Furazolidone Treatment of Experimental Pullorum Disease in Adult Chickens

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IT HAS BEEN reported by Gordon and Tucker,[2] working with adult pullorum reactors obtained by field pullorum testing, that access to feed containing 0.02 or 0.04 per cent of furazolidone resulted in a diminution of the agglutination titer and a reduction of the number of birds from which *Salmonella pullorum* could be isolated, as compared with untreated controls. Wilson,[4] working with artificially infected birds, failed to alter the agglutination titers but recovered *S. pullorum* or *Salmonella gallinarum* from a larger proportion of the untreated birds than from the treated birds which had received 0.04 per cent of furazolidone in the feed. Gordon and Tucker,[3] using reactors to both field testing and artificial *S. pullorum* infections, found that the birds fed 0.04 per cent furazolidone in the mash showed a reduction in agglutination titer and a lower recovery rate over the birds which were not treated.

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The following experiment was planned to determine whether the use of furazolidone at the 0.011 per cent level in the feed, for pullorum infection, would interfere with the results of agglutination testing or the cultural procedures recommended in the National Poultry Improvement Plan.[1]

MATERIALS AND METHODS

Thirty-nine, 6-month-old crossbred chickens were inoculated intramuscularly with 1 ml. of a 48-hour broth culture of an intermediate strain of *S. pullorum* isolated from specimens submitted to the Animal Disease Diagnostic Laboratory of Purdue University. Starting 15 days after inoculation and continuing for two weeks, 17 females and 3 males were fed a commercial all-mash ration containing NF 180 * at the rate of 2 lb. per ton (0.011% furazolidone) which was the only medicament. As controls, 16 females and 3 males were kept on the same diet without the drug throughout the experiment. When furazolidone was not being fed, the birds were allowed to cohabit on deep corncob litter. Eighty-six days after the inoculation with *S. pullorum*, the former control birds were fed the same commercial mash containing 2 lb. of NF 180 per ton for 46 days, thus becoming the treated group in the second experiment. The

* Supplied through the courtesy of Hess and Clark, Inc., Ashland, Ohio.

TABLE 1—Agglutination Titers Developed in Birds Inoculated with Salmonella Pullorum (Experiment 1)

Agglutination titer	No. of birds with various agglutinin titers at days in relationship to start of treatment										
	−3	+4	+11	+18	+25	+32	+39	+46	+53	+60	+67 days
Treated birds (20)											
1:800	15	6	....	....	....	....	....	....	....	....	....
1:400	2	8	11	5	1	1	1	....	3	2	2
1:200	1	4	5	7	5	1	1	3	2	2	1
1:100	2	1	3	5	3	8	10	5	9	2	4
1:50	....	1	1	3	8	5	3	6	3	6	5
1:25	....	....	....	....	3	4	3	4	1	5	4
1:10	....	....	....	....	....	1	2	....	....	....	1
None	....	....	....	....	....	....	....	2	2	3	3
Nontreated birds (18)											
1:800	15	15	4	2	1	1	....	....	1	2	1
1:400	1	2	11	12	8	3	3	5	4	1	2
1:200	....	....	....	2	3	4	3	3	4	5	6
1:100	1	1	2	1	3	4	6	4	5	2	....
1:50	....	....	1	1	2	3	3	2	2	5	3
1:25	1	....	....	....	1	3	1	2	....	2	3
1:10	....	....	....	....	....	....	2	2	2	1	1
None	....	....	....	....	....	....	....	....	....	....	1

former treated group was not given furazolidone during this period and, thus, became the control.

Blood samples were taken from the birds 12 days after they were infected with *S. pullorum* and weekly, thereafter, for ten weeks. In the second experiment, the samples were taken 11 days after the treatment was started in one group and weekly, thereafter, for six weeks. Tube-agglutination tests for *S. pullorum* antibodies were conducted on all serums with a pullorum antigen made with strains 17, 19, and 20. The dilutions of serum used were 1:10, 1:25, and twofold dilutions to 1:800. In reading the agglutination tests, complete sedimentation of the bacterial cells was considered positive; incomplete clearing or no clearing were considered negative.

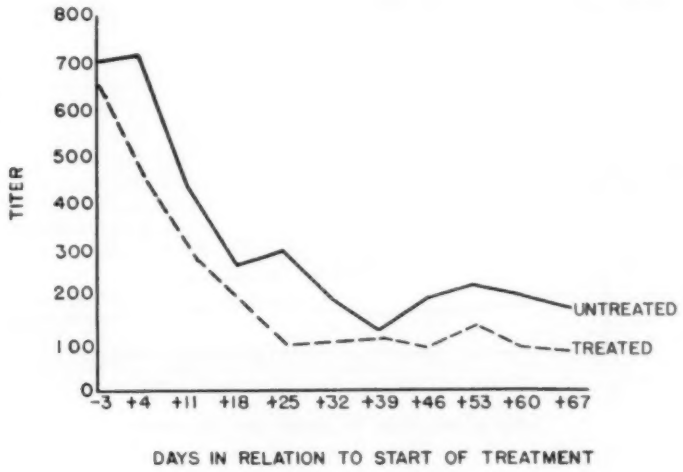
After the last sampling, all birds were given the ration without furazolidone for 72 hours and then killed. The heart and pericardium, a piece of the liver, the emptied gallbladder, spleen, pancreas, ovary (except large, normal looking ova) or testis, and a piece of kidney, crop, and intestines, including both cecal tonsils, were retained for bacteriological examination. These tissues were frozen at —20 C. and stored for four to seven weeks. As time became available, the tissues were thawed at room temperature and bacteriological cultures made. The crop and intestines were put into selenite broth (Difco). The other organs were ground in 100 ml. nutrient broth (Difco) enriched with 3 per cent yeast extract (Difco); 10-ml. portions of this were added to 100 ml. nutrient broth, enriched with 3 per cent yeast extract and selenite broth (Difco). After 16 hours' incubation at 37 C., brilliant green agar (Difco) and tryptose agar (Difco) plates were inoculated from the broth cultures. When typical colonies were not obtained from the first plating, the broths were replated after 24 more hours' incubation at room temperature. The agar plates were incubated for 24 hours at 37 C. Final identification of *S. pullorum* was made with carbohydrate broths and other appropriate tests.

RESULTS

In the first experiment, 2 birds died in the nontreated group, 1 from visceral lymphomatosis and 1 from unknown causes. The first bird was not included in the results but *S. pullorum* titers were obtained from the latter bird at all bleedings except the last, so it was included in the first experiment. In the second experiment, 1 nontreated bird was found in the pen with the treated birds and was not included in the results.

The number of birds with each *S. pullorum* titer at each blood sampling, following two weeks' treatment with furazolidone, is shown (table 1) as is the average titer over the same period (graph 1). It can be seen that the titer against the *S. pullorum* antigen dropped slightly faster in the treated than in the nontreated group. However, the titers in both groups dropped at approximately the same rate. In the treated group, 8 birds became negative; that is, they had titers of less than 1:50 (1:50 is the diagnostic dilution for chickens recognized by National Poultry and Turkey Improvement Plans [1]), 3 of these being negative even in the 1:10 dilution. In the nontreated group, 6 birds became negative with 2 showing no demonstrable antibodies.

The results of the second experiment are shown (table 2, graph 2). In this case, the



Graph 1—Average weekly agglutination titers—experiment 1.

TABLE 2—Agglutination Titers Developed in Birds Inoculated with Salmonella Pullorum (Experiment 2)

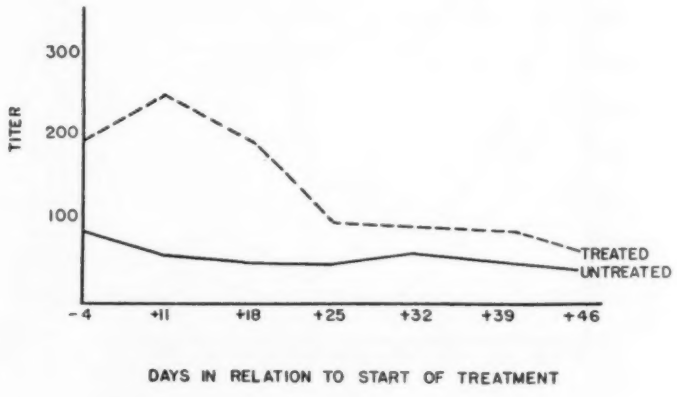
Agglutination titer	No. of birds with various agglutinin titers at days in relationship to start of treatment							*S. pullorum* recovered
	−4	+11	+18	+25	+32	+39	+46 days	
				Treated birds (17)				
1:800	1	2	1	....	....	....	....	....
1:400	2	4	3	1	....	....	....	....
1:200	6	1	4	3	1	3	1	....
1:100	....	4	2	3	2	5	5	2
1:50	3	1	2	4	5	3	4	3
1:25	3	3	2	2	2	1	1	....
1:10	1	1	2	3	3	3	1	....
None	1	1	1	1	2	2	5	....
				Nontreated birds (19)				
1:800	....	....	....	....	....	....	....	....
1:400	2	....	....	....	....	....	....	....
1:200	1	2	....	1	1	....	....	....
1:100	4	7	6	4	6	5	2	1
1:50	4	3	4	5	5	6	7	3
1:25	4	3	3	2	....	1	2	....
1:10	1	1	3	3	1	2	2	1
None	3	3	3	4	6	5	6	....

S. pullorum titer of birds in the treated group rose slightly during the first week. After this period, the titers in both groups dropped until the average in the treated group was only slightly higher than that in the nontreated group. At this time, the treated group contained 7 birds which became negative when 1:50 is considered the diagnostic dilution; 5 of the 7 birds had no demonstrable antibodies. The nontreated group had 10 birds with titers less than 1:50, which would be considered negative; 6 of the 10 had no demonstrable antibodies.

Salmonella pullorum was recovered from 5 birds in each group. In the treated group in the second experiment, the organism was recovered from 2 birds with *S. pullorum* agglutination titers of 1:100 and from 3 birds with a titer of 1:50. In the control group, the organism was recovered from 1 bird with a titer of 1:100, 3 birds with titers of 1:50, and 1 bird with a titer of 1:10.

DISCUSSION

It is of interest that the titers recorded in the first column of table 1 and the start of graph 1 were obtained with blood drawn 12 days after infection and three days before treatment. At the second sampling, four days after treatment, there were rises in the titers of the birds which were not given the treatment. From this point to 67 days after treatment, the difference in the titers remained approximately the same with the titers in both groups dropping at the same rate. As titer levels de-



Graph 2—Average weekly agglutination titers—experiment 2.

creased, the rate of decline tended to decrease. Since the furazolidone treatment was associated with diminution of agglutinin titers only in the case of the initial infection, it seems likely that this may have been caused by interruption of the initial phase of the disease. Following this, the agglutinin titers showed no alteration attributable to the treatment.

TABLE 3—Analysis of Variance of Agglutination Titers Obtained in Experiment 1

Source of variation	Degrees of freedom	Mean square	F ratio
Treatment	1	5.83	4.70*
Error (1)	36	1.24	.....
Weeks	10	5.06	60.31†
Weeks x treatments	10	0.121	1.44
Error (2)	356	0.084	.....

* Significant ($P \leq .05$); † highly significant ($P \leq .01$).

An analysis of variance was made on the titers produced during the first experiment. The data were converted to logarithms as the dilutions used in the experiment resulted in a geometric progression rather than an arithmetic progression. Also, for statistical purposes, all the titers recorded as negative were considered as positive in the 1:5 dilution. The results of analysis of variance (table 3) indicate that the furazolidone and control treatments were significantly different ($P \leqslant 0.05$) and that the treatment by week interaction was not significant. The treatment difference being significant but not changing with time, as indicated by the interaction not being significant, supports the contention that after the original infection furazolidone did not interfere with blood titers.

In the experiment in which the birds received continuous furazolidone treatment, the *S. pullorum* titers of the treated group increased for the first week, dropped during the next two weeks, and then showed little change. The treated birds continued to maintain higher titers than the control birds. Since the dilution of 1:50 has become the standard diagnostic dilution for identifying *S. pullorum* reactors, it is of interest that, after 46 days on feed containing furazolidone, the treated birds still had an average positive titer above 1:50. Only 7 of 17 birds had *S. pullorum* titers of less than 1:50 while in the nontreated group, 10 of 19 birds had agglutinin titers of less than 1:50 and, in routine testing, would be considered negative. This indicates that the use of furazolidone had no significant effect on the titers produced by *S. pullorum* except, perhaps, during the bacteremia.

With the techniques used, *S. pullorum* was recovered from 5 birds in each group. In the group treated for 46 days, beginning 86 days postinoculation, the organism was recovered from 5 (50%) of 10 birds which had titers of 1:50 or greater and would be positive in routine testing. In the untreated group, the organism was recovered from 4 (44%) of 9 birds which had titers of 1:50 or greater. *Salmonella pullorum* was recovered from 1 bird with a titer of 1:10. However, there were 9 other birds in this group and 7 in the treated group with titers less than 1:50 from which *S. pullorum* was not recovered. The ability to isolate *S. pullorum* was more closely related to the final agglutination titer of the individual birds than to the use of furazolidone.

SUMMARY AND CONCLUSIONS

Furazolidone, when used at 0.011 per cent of the feed, did not materially affect the agglutinin titers engendered among adult chickens artificially infected with *Salmonella pullorum*, except perhaps during the bacteremia. The treatment did not affect the recoverability of the bacterium.

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A Comparison of the Indirect Complement-Fixation Test, the Direct Complement-Fixation Test, and the Macroscopic-Agglutination Test for Ornithosis Antibodies in Turkey Serums

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THE STATUS of ornithosis (psittacosis) in the United States was recently reviewed by Andrews [1] who pointed out that turkeys have received an increasing amount of attention as a reservoir of ornithosis. More than 300 cases have been reported in man, with at least seven deaths, due to association with the disease in turkeys during the years 1948 to 1956. Agents considered to be ornithosis virus have been isolated from turkeys in seven states.

Information concerning the extent and location of ornithosis among domestic turkeys is not presently available. A reliable and economical method of detecting the infection is needed. The indirect complement-fixation (ICF) test [4] is the only generally accepted serological test for ornithosis in turkeys. This test requires complex, time-consuming procedures and pigeon or human anti-ornithosis serum of high titer. This antiserum has been unobtainable in quantities sufficient for extensive survey purposes.

The purpose of this paper is to report a comparison of the results using the ICF test, a direct complement-fixation (DCF) test employing the detergent-extracted antigen as suggested by Benedict,[2] and a macroscopic-agglutination test as suggested by Labzoffsky,[7] using 923 turkey serums.

TABLE 1—Direct Complement-Fixation, Indirect Complement-Fixation, and Agglutination Titers of Ornithosis Antibodies of 923 Turkey Serums

Group 1—89 experimentally infected turkeys

Test	Serum dilutions 1:2	1:4	1:8	1:16	1:32	1:64	Total positive
Agglutination	4	26	31	8	1	0	70
Direct complement-fixation	38	19	6	2	0	0	65
Indirect complement-fixation	0	28	20	14	6	0	68

Group 2—183 noninfected turkeys

Test	Serum dilutions 1:2	1:4	1:8	1:16	1:32	1:64	Total positive
Agglutination	90	26	1	0	0	0	117
Direct complement-fixation	0	1	1	0	0	0	2
Indirect complement-fixation	0	0	0	0	0	0	0

Group 3—651 serums (farm flocks)

Test	Serum dilutions 1:2	1:4	1:8	1:16	1:32	1:64	Total positive
Agglutination	196	113	102	12	0	0	423
Direct complement-fixation	37	23	1	0	0	0	61
Indirect complement-fixation	60	51	11	2	0	0	124

MATERIALS AND METHODS

The turkey serums were from three groups of turkeys. Group 1 consisted of mature turkey hens that had been artificially infected with ornithosis. Group 2 was composed of partially mature turkeys

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of both sexes that had been hatched and raised in isolation and had shown no evidence of ornithosis. Group 3 represented farm flocks of unknown history. The three tests were made concurrently on each serum.

The yolk sac antigen used in the indirect test was prepared according to the method described by Karrer *et al.*[5]

The detergent-extracted antigen used in the DCF test was prepared according to the method described by Benedict.[3]

The agglutination test antigen was a preparation of stained, 6BC strain of psittacosis elementary bodies. The virus was propagated in embryonating chicken eggs by the same procedures used in preparation of yolk sac antigen. The agglutination test antigen was processed by harvesting yolk sac and allantoic fluid separately from chicken embryos which died four days after inoculation. The yolk sacs were ground in a blender and 500 units of penicillin and 1 mg. of streptomycin were added to each milliliter of the ground suspension. The yolk sac tissues in suspension were digested by 0.5 per cent trypsin at 37 C. for 48 hours. Formaldehyde (0.2%) was added to both the digested yolk sac suspension and the allantoic fluid, which were then stored at 4 C. for six days. The suspension was then centrifuged at 12,500 x g for one hour, the supernatant fluid discarded, and the pellet resuspended in buffered water. Differential centrifugation and washing were repeated until the material contained elementary bodies of relatively uniform size as determined by microscopic examination of a stained portion.

After processing, the elementary bodies from the yolk sac and allantoic fluid were pooled and the resulting suspension standardized to the density of a McFarland nephalometer tube No. 8. One drop of 2 per cent aqueous safranine was added to each 5.0 ml. of the standardized suspension which was then stored at 4 C. After 24 hours, the stained elementary bodies were again deposited by centrifugation and resuspended to standardized volume in buffered water. This suspension constituted the test antigen.

The complement, hemolysin, and sheep erythrocytes were secured and employed in the manner commonly used in the conventional DCF test. The dilutions of serum, complement, and hemolysin and suspensions of sheep erythrocytes used in the tests were made in veronal buffered saline solution prepared according to the method described by Karrer *et al.*[5] Buffered water (PH 7.0), used for washing and suspending elementary bodies for agglutination antigen, was prepared according to the method described by Kolmer, Spaulding, and Robinson.[6]

The anti-ornithosis serum used in the ICF test was of human origin and was secured from the Texas State Department of Health.

The ICF test was conducted according to the method described by Karrer *et al.*[5]

The DCF test was performed by the conventional method, using 4 units of antigen, 2 exact units of complement, 2 units of hemolysin, and a 2 per cent suspension of sheep erythrocytes in 0.25-ml. amounts. Serial twofold dilutions of serums were tested. Diluted serum, complement, and antigen were mixed and incubated at 37 C. for 75 minutes.

TABLE 2—Comparison of Results of Indirect Complement-Fixation Test, Direct Complement-Fixation Test, and Agglutination Test, by Pairs, Showing Agreements and Discrepancies in Numbers and Percentages of Serums Tested

	Group 1 serums		Group 2 serums		Group 3 serums		Total serums	
	(No.)	(%)	(No.)	(%)	(No.)	(%)	(No.)	(%)
Pos. ICF–pos. DCF	52	58.4	0	.....	39	5.9	91	9.9
Neg. ICF–neg. DCF	8	8.9	181	98.9	505	77.7	694	75.2
Total agreeing	60	67.3	181	98.9	544	83.6	785	85.1
Neg. DCF–pos. ICF	16	17.9	0	.....	85	13.0	101	10.9
Pos. DCF–neg. ICF	13	14.7	2	1.1	22	3.4	37	4.0
Total disagreeing	29	32.6	2	1.1	107	16.4	138	14.9
Pos. aggl.–pos. ICF	53	59.6	0	.....	82	12.6	135	14.6
Neg. aggl.–neg. ICF	4	4.4	66	36.0	186	28.6	256	27.7
Total agreeing	57	64.0	66	36.0	268	41.2	391	42.3
Neg. aggl.–pos. ICF	15	16.9	0	.....	42	6.4	57	6.1
Pos. aggl.–neg. ICF	17	19.1	117	64.0	341	52.6	475	51.5
Total disagreeing	32	36.0	117	64.0	383	59.0	532	57.6
Pos. aggl.–pos. DCF	54	60.7	1	0.6	39	5.9	94	10.2
Neg. aggl.–neg. DCF	8	8.9	65	35.4	206	31.7	279	30.2
Total agreeing	62	69.6	66	36.0	245	37.6	373	40.4
Neg. aggl.–pos. DCF	11	12.4	1	0.6	22	3.4	34	3.6
Pos. aggl.–neg. DCF	16	17.9	116	63.4	384	58.9	516	55.9
Total disagreeing	27	30.3	117	64.0	406	62.3	550	59.5

Pos. = positive; neg. = negative; ICF = indirect complement-fixation; DCF = direct complement-fixation; aggl. = agglutination test.

The hemolytic system was then added and the test incubated an additional 30 minutes.

The macroscopic-agglutination test was performed by placing 0.02 ml. of undiluted and two-fold dilutions of each serum on a glass plate. An equal quantity of antigen was mixed with each serum and the test was read in eight minutes. Reactions were recorded as 4 plus for coarse clumping, 3 plus for moderate clumping, 2 plus for slight clumping, and 1 plus for the least detectable clumping of the antigen. No detectable clumping was recorded as 0.

In the agglutination and the DCF tests, 4 plus and 3 plus reactions were considered positive. In the ICF test, 0 or 1 plus reactions were considered positive. All other reactions in all tests were considered negative. A serum reacting positively at any dilution in any test was considered to be a positive serum.

RESULTS

Reactions to the three tests for ornithosis antibodies in 923 turkey serums in dilutions of 1:2 to 1:64 are summarized (table 1). Results are compared by pairs of tests (table 2) and by combinations of reactions to the tests (table 3).

Agreement among the three tests as a group was poor. Only 34.3 per cent of all serums tested were determined to be either positive or negative by all three tests. Eight combinations of reactions to the tests were observed, which indicates that each test reflected a different serum-antigen reaction (table 3).

TABLE 3—Combinations of Reactions to the Three Serological Tests for Ornithosis Antibodies by Turkey Serums with Number and Percentage of Serums Reacting in Each Combination

Agglutination test	ICF test	DCF test	Serums (No.)	Serums (%)
Positive	Positive	Positive	72	7.8
Positive	Positive	Negative	63	6.8
Positive	Negative	Negative	451	48.8
Positive	Negative	Positive	24	2.6
Negative	Negative	Negative	244	26.5
Negative	Negative	Positive	12	1.3
Negative	Positive	Positive	21	2.2
Negative	Positive	Negative	36	3.8

DCF = direct complement-fixation; ICF = indirect complement-fixation.

DISCUSSION

The large number of serums that reacted positively to the agglutination test and negatively to the other two tests seemed to indicate that the antigen used in the agglutination test was subject to some action of turkey serum unrelated to ornithosis infection (table 1).

The ICF test and the DCF test were in agreement on 85 per cent of all serums tested in classifying the serums as positive or negative (table 2). A part of the discrepancy between these two tests undoubtedly was due to the greater sensitivity of the indirect test.

On the basis of these comparisons, further study of the DCF test, employing detergent-extracted antigen, seems justified. This test should not be evaluated strictly on the basis of its correlation with the ICF test because it is not known whether the two tests measure the same antigen-antibody reaction. The ICF test failed to detect antibodies in 21 of 89 serums from birds known to be infected (table 1).

Recently, turkey serums from group 2, obtained and studied three months later, have shown a number of positive reactions to the ICF test with titers of 1:2, 1:4, 1:8, and 1:16 in the absence of any known ornithosis infection. Whether these reactions are due to maturity, to some inapparent infection, or to other factors remains to be determined. These findings would seem to parallel the observations from testing large numbers of farm flocks which also had no history of ornithosis.

SUMMARY

Serums of 923 turkeys were tested for ornithosis antibodies by the macroscopic-agglutination test, the indirect complement-fixation (ICF) test, and the direct complement-fixation (DCF) test.

The correlation between the results of the agglutination test and the ICF test was 42.3 per cent. Agreement between the results of the agglutination test and the DCF test was 40.4 per cent.

The ICF test and the DCF test gave similar results on 85.1 per cent of the serums tested.

Turkeys that reacted negatively to the ICF test in these comparisons, reacted positively three months later without showing clinical evidence of ornithosis infection.

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Growth and Regression in Chickens of a Duck Variant of the Rous Sarcoma

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DURAN-REYNALS[2] demonstrated that a duck variant, 14(d)7, of the Rous sarcoma, although highly virulent for the duck, was unable to grow progressively except in young hosts. Furthermore, this variant had the unusual property of inducing tumors in all the species on which it had been tried, e.g., chickens, ducks, turkeys,[5] and pigeons.[1] Both its virulence and its broad host range may indicate that this tumor virus, 14(d)7, has a greater antigenic complex than the viruses of other Rous sarcoma tumor strains.

Therefore, it was considered of interest to determine whether regression of variant 14(d)7 would occur as readily in chickens, using a large number, as in ducks. Since it is known that there is a serological relationship between the virus of the Rous sarcoma and that of visceral lymphomatosis,[3,4] the regression of the duck variant in chickens suggests the possibility of using this virus for immunization against malignant conditions, especially that of avian lymphomatosis.

MATERIALS AND METHODS

Duck variant 14(d)7 has been kept since 1947 by cell passages through ducklings and chicks. In the present study, tumors grown in ducklings for about two weeks were processed with buffered saline solution to obtain cell suspensions of different concentrations which were injected superficially into the breasts of chickens.

The chickens, which were White Rocks from a strain maintained at the Department of Animal and Poultry Industry of the University of Delaware, were inoculated at 10 weeks of age. Throughout the experiment, they were kept separate from the controls.

RESULTS

Experiment 1.—The tumor was frozen for 20 hours at −65 to −70 C. before being used. Cell suspensions were diluted at 1:20, 1:200, and 1:2,000, and 0.1 ml. of each dilution was injected into the breast muscles of 20 chickens, 10 weeks of age. All birds developed tumors which often reached sizes up to 7.5 cm. All tumors except one regressed within two months after injection. The exception was in a bird that died as a result of the injected tumor (1:2,000 dilution).

Experiment 2.—The tumor used was secured from freshly killed ducklings. The tumor cells (0.1 ml. of a dilution of 1:200) were injected into the breast muscles of 150 chickens, 10 weeks old. All the chickens injected developed large tumors but, contrary to the observations in the first experiment, a number of these tumors grew steadily, with the result that 59 birds died of generalized sarcomas during the second month after tumor inoculation. and 4 died within another five months. However, shortly after injection, the test and control birds were exposed to a severe form of chronic respiratory disease, complicated with Newcastle disease, which caused the deaths of 7 of the test birds. In the remaining 80 chickens, the tumor underwent total regression.

SUMMARY

Tumors were induced in chickens with duck variant 14(d)7 of the Rous sarcoma. These tumors frequently regressed. The influence of the amount of inoculum given on tumor regression, as well as the possible application of 14(d)7 for immunization against naturally occurring lymphomatosis, is being studied.

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Infectivity of Colonial Variants of *Vibrio Fetus* Strains for the Chicken Embryo

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THE INFECTIVITY of *Vibrio fetus* for laboratory animals has been studied by several investigators;[3, 6, 7, 10-12, 14, 15] however, relatively little attention has been given to the infectivity of this organism for the chicken embryo.

Plastridge and Williams[8] reported that several 12-day-old chicken embryos inoculated with a *V. fetus* culture of bovine origin died within five days. Webster and Thorp[16] propagated *V. fetus* through 23 serial passages in embryonating chicken eggs and described the lesions produced in this host.

Herzberg and Ristic[4] observed a number of colonial variants in *V. fetus* strains when cultures were plated on solid medium and observed by oblique, indirect, transmitted light. Ristic, Herzberg, and Sanders[9] further examined stock cultures of *V. fetus* of bovine, ovine, and human origin, using the above technique, and described the presence of one smooth and four distinct nonsmooth colonial types with correlated cell morphology. These authors also described antigenic and metabolic differences between the smooth and nonsmooth cultures under examination.

In addition to the above described colonial variants of *V. fetus* strains, the existence of a stable "cut-glass" colonial type in a noncatalase producing "saprophytic" Vibrio has been observed by Ristic, White, and Doty.[13] The latter Vibrio has been described by Bryner and Frank[1] as not being incriminated with abortions and infertility of cattle.

Within several bacterial genera, e.g. Brucella and Salmonella, the smooth to rough dissociation has been characterized by concomitant changes in virulence. This experiment was designed to investigate the infectivity of smooth and rough colonial variants of *V. fetus* strains of bovine, ovine, and human origin and the noncatalase-producing saprophytic Vibrio for the chicken embryo. It was thought that such a study might provide a means of evaluating the relative pathogenicity of *V. fetus* and related Vibrio strains of diverse origin as has been shown to some extent in studies with laboratory animals.

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MATERIALS AND METHODS

Five of the strains used (UF, F, 53-D, I, and 1134) were typical representatives of *V. fetus* on the basis of their morphological, cultural, and biochemical characteristics. The smooth colonies of these strains were bluish, raised, entire-edged, translucent, and contained very little internal granulation. The rough colonies of the above strains were grayish white and opaque, occasionally with irregular surfaces. They were of dry consistency and tended to break apart when pushed with a loop. Strain V, of human origin, was observed only in the smooth form and differed from the above described smooth *V. fetus* form by the possession of a secondary colony on many of the colonies. The secondary colony was distinguished by being centrally located, elevated, and darker in color than the parent colony. In addition, catalase-negative and H_2S-positive strain 4220, which was in a stable cut-glass colonial form, was employed in this study. The stable cut-glass colonial form has been characteristic of several other non-catalase-producing strains of bovine origin. Cultures of this group were isolated as large, round, occasionally irregular edged, raised colonies with entire cut-glass surfaces or yellowish centers and cut-glass peripheries, mucoid in consistency. Histories of the seven strains used are given (table 1).

Strains UF, 53-D, 1134, V, and 4220 had been maintained for varying periods by monthly transfer in Brucella broth,* to which 0.16 per cent agar was added. Strains I and F were lyophilized shortly after isolation and were reactivated for study.

The smooth and rough *V. fetus* variants were selected by plating from Brucella semisolid cultures (0.16% agar) to Brucella agar.* The agar content of the Brucella solid medium was increased to 2 per cent, in order to obtain more discrete colonies. All cultures were incubated for 48 hours at 37 C. in an atmosphere containing 15 per cent CO_2. Colonies were examined under 10 to 45 x magnification using oblique, transmitted light, according to the method of Henry.[5] Repeated selections of single smooth or rough colonies from subcultures were often necessary in order to obtain the desired variant type. The plate growth, consisting of pure smooth or rough variants, was washed with sterile 0.85 per cent sodium choride solution and the resulting suspension was used to inoculate Brucella agar in 16-oz. prescription bottles. Duplicate inoculums were

* A product of Albimi Laboratories, Inc., Brooklyn, N. Y.

TABLE 1—Histories of Vibrio Strains Studied

Strain	Origin	Date of isolation	Recovered by
UF	Bovine aborted fetus, Fla.	1954	M. Ristic
F	Bovine cervical mucus, Fla.	1955	F. H. White
53-D	Ovine aborted fetus, Colo.	1952	W. R. Graham
I	Ovine aborted fetus, Ill.	1951	H. E. Rhoades
1134	Human blood, Mass.	1953	Peter Bent, Brigham Hospital *
V	Human blood, France	1949	R. Vinzent †
4220	Bovine vaginal mucus, Md.	1956	A. H. Frank

* Received from Miss E. O. King, Communicable Disease Center, U. S. Public Health Service, Atlanta, Ga.
† Received from B. D. Firehammer, Montana State College, Bozeman.

carried on Brucella agar plates to ascertain the colonial purity of the respective variants.

Following incubation, growth in the bottles was washed off with sterile 0.85 per cent sodium chloride solution and smears of the harvested suspension of *V. fetus* cells were examined for purity by Gram's stain. The cell suspension was filtered through sterile glass wool, and the resulting filtrate was standardized to a density of 35 per cent light transmission in a Leitz photoelectric colorimeter, using a No. 550 green filter. Serial tenfold dilutions from 10^{-0} through 10^{-8} were prepared using sterile 0.85 per cent sodium chloride solution as the diluent. Plate counts were made on Brucella agar, using suitable dilutions, and 4, 10-day-old chicken embryos were inoculated with 0.2 ml. of each dilution via the allantoic cavity. Five to 10 uninoculated embryos from the same egg setting were incubated simultaneously in order to provide embryo controls. In addition, 4 embryos from each setting were inoculated with 0.2 ml. of sterile 0.85 per cent sodium chloride solution.

Chicken embryos were incubated at 37 C. and the eggs were candled daily. Embryos which died during the first six days after inoculation were cultured by streaking a 5-mm. loopful of allantoic fluid on Brucella agar. All chicken embryos alive after the sixth day of incubation were cultured as above. All embryos were examined for gross lesions and their livers, viscera, and membranes were examined for histological abnormalities. The colonial types of *V. fetus* isolated from both dead and living chicken embryos were determined as previously described.[5, 9]

RESULTS AND DISCUSSION

Chicken Embryo Mortality.—Total embryo mortality (table 2) varied from 10.7 per cent in the case of UF smooth variant (bovine) and F rough variant (bovine) strains to 85.7 per cent in the case of 1134 smooth variant (human) strain. There were no deaths among uninoculated control embryos or among embryos inoculated with sterile 0.85 per cent sodium chloride solution. Although wide variation in mortality was demonstrated among strains and variants, smooth variants of strains F, 53-D (ovine), and 1134 were more lethal for chicken embryos than were the rough variants of these strains. On the other hand, the rough variant of strain UF induced more deaths than did the smooth variant of the homologous culture. Differences in mortality, demonstrated with smooth and rough variants of I strain, in which the rough variant induced a higher

TABLE 2—Infectivity of Colonial Variants of Vibrio Fetus Strains for Chicken Embryos—Mortality and Isolation of Variants

Species origin	Variant strain	Viable cells inoc. at 10^{-0}	Total mortality * †	Recoveries—dead embryos ‡	Recoveries—live embryos §	Total recoveries	Variant type recovered
Bovine	UF (S)	2.31×10^8	3/28 (10.7%)	2/3 (66.6%)	5/25 (20.0%)	7 (25.0%)	S + M
	UF (R)	1.88×10^8	14/28 (50.0%)	5/14 (35.7%)	2/14 (14.2%)	7 (25.0%)	R + M
	F (S)	6.62×10^8	9/28 (32.1%)	3/9 (33.3%)	0/19	3 (10.7%)	S + M
	F (R)	3.20×10^7	3/28 (10.7%)	1/3 (33.3%)	0/25	1 (3.5%)	R
Ovine	53-D (S)	3.91×10^8	17/28 (60.7%)	6/17 (35.2%)	1/11 (9.1%)	7 (25.0%)	S + M
	53-D (R)	1.02×10^9	9/28 (32.1%)	2/9 (22.2%)	1/19 (5.2%)	3 (10.7%)	R + M
	I (S)	3.10×10^7	6/28 (21.4%)	3/6 (50.0%)	5/22 (22.7%)	8 (28.5%)	S + M + R
	I (R)	2.06×10^8	20/28 (71.4%)	17/20 (85.0%)	4/8 (50.0%)	21 (75.0%)	S + R + M
Human	1134 (S)	4.74×10^8	24/28 (85.7%)	3/24 (12.5%)	0/4	3 (10.7%)	S + CG
	1134 (R)	2.36×10^9	11/28 (38.5%)	1/11 (9.1%)	0/17	1 (3.5%)	R
	V (S)	4.10×10^8	8/28 (28.5%)	8/8 (100.0%)	9/20 (45.0%)	17 (60.7%)	S
Bovine "saprophytic"	4220 (CG)	1.04×10^8	18/28 (64.2%)	8/18 (44.4%)	0/10	8 (28.5%)	CG

* From inoculation of dilutions 10^{-0} through 10^{-8}, 4 embryos per dilution. † Numerator, number dead; denominator, number inoculated. ‡ Numerator, number recovered; denominator, number dead. § Numerator, number recovered; denominator, number alive after six days. S = Smooth variant; R = rough variant; M = mucoid variant; CG = cut-glass variant.

TABLE 3A—Infectivity of Colonial Variants of Vibrio Fetus Strains for Chicken Embryos—Relation of Number of Viable Cells Inoculated to Embryo Deaths

Strain	UF (bovine)				53-D (ovine)				1134 (human)			
Variant	S		R		S		R		S		R	
Dilutions *	Viable cells	Mortality	Viable cells	Mortality	Viable cells	Mortality	Viable cells	Mortality	Viable cells	Mortality	Viable cells	Mortality
10^{-0}	2.31×10^{8}	0	1.88×10^{8}	2	3.91×10^{8}	2	1.04×10^{9}	4	4.74×10^{8}	3	2.36×10^{8}	0
10^{-1}	2.31×10^{7}	0	1.88×10^{7}	1	3.91×10^{7}	3	1.04×10^{8}	2	4.74×10^{7}	4	2.36×10^{7}	1
10^{-2}	2.31×10^{6}	0	1.88×10^{6}	2	3.91×10^{6}	4	1.04×10^{7}	0	4.74×10^{6}	4	2.36×10^{6}	1
10^{-3}	2.31×10^{5}	0	1.88×10^{5}	2	3.91×10^{5}	2	1.04×10^{6}	1	4.74×10^{5}	2	2.36×10^{5}	1
10^{-4}	2.31×10^{4}	2	1.88×10^{4}	1	3.91×10^{4}	3	1.04×10^{5}	2	4.74×10^{4}	4	2.36×10^{4}	1
10^{-5}	2.31×10^{3}	1	1.88×10^{3}	2	3.91×10^{3}	1	1.04×10^{4}	0	4.74×10^{3}	4	2.36×10^{5}	3
10^{-6}	2.31×10^{2}	0	1.88×10^{2}	4	3.91×10^{2}	2	1.04×10^{3}	0	4.74×10^{2}	3	2.36×10^{3}	4
Total mortality		3		14		17		9		24		11

* Four embryos inoculated with each dilution; S = smooth variant; R = rough variant.

incidence of deaths, can not be considered significant due to considerable *in vivo* dissociation of the inoculated variants. That is, inoculated R and S variants of I strain upon recovery revealed the presence of smooth, rough, and mucoid colonial types. The saprophytic strain 4220 produced a mortality of 64.2 per cent of inoculated chicken embryos. This was a higher mortality than that produced by *V. fetus* variant strains, with the exception of smooth variant 1134 and dissociated rough variant of I strain.

With certain exceptions, smooth variants of bacterial strains are generally considered more pathogenic for natural hosts, as well as for susceptible laboratory animals, than are rough variants. According to the data presented, this held true with three paired variants (strains F, 53-D, and 1134), but not in the case of UF strain. This may indicate that the colonial type of *V. fetus* is not the only factor determining its pathogenicity for chicken embryos.

Pathology.—Varying degrees of inflammatory reactions were revealed in most instances upon gross examination of dead embryos. Dermal congestion was most readily observed. Enlargement of the liver, with yellowish brown discoloration, was observed in most of the embryos examined. Microscopic sections showed intense congestion of the membranes and of the viscera of the embryos. Extensive thrombosis of the chorioallantoic vessels and hemorrhages of the hepatic tissue were prominent findings. These findings were similar to those described in more detail elsewhere.[16]

Recoveries of Vibrio Fetus.—Recoveries of *V. fetus* from dead embryos (table 2) varied from 9.1 per cent with strain 1134 (R) to 100 per cent with strain V (S). With certain exceptions, *V. fetus* was cultured more frequently from embryos inoculated with smooth variants than from those inoculated with rough variants.

Vibrio fetus was recovered from embryos which were alive six days after inoculation in the cases of seven of the variant strains used. Gross pathological lesions were not observed in these embryos. This phenomenon apparently resulted from a host-parasite interaction in which either the embryo resistance was increased or the pathogenicity of the organism was de-

TABLE 3B—Infectivity of Colonial Variants of Vibrio Fetus Strains for Chicken Embryos—Relation of Number of Viable Cells Inoculated to Embryo Deaths

Strain	F (bovine)				I (ovine)				V. (human)		4220 bovine saprophytic	
Variant	S		R		S		R		S		CG	
Dilutions *	Viable cells	Mortality	Viable cells	Mortality	Viable cells	Mortality	Viable cells	Mortality	Viable cells	Mortality	Viable cells	Mortality
10^{-0}	6.62×10^{8}	1	3.2×10^{7}	1	3.1×10^{7}	1	2.06×10^{8}	4	4.1×10^{6}	3	1.04×10^{8}	4
10^{-1}	6.62×10^{7}	1	3.2×10^{6}	0	3.1×10^{6}	0	2.06×10^{7}	2	4.1×10^{5}	0	1.04×10^{7}	3
10^{-2}	6.62×10^{6}	2	3.2×10^{5}	1	3.1×10^{5}	2	2.06×10^{6}	4	4.1×10^{4}	0	1.04×10^{6}	2
10^{-3}	6.62×10^{5}	2	3.2×10^{4}	0	3.1×10^{4}	1	2.06×10^{5}	4	4.1×10^{3}	3	1.04×10^{5}	2
10^{-4}	6.62×10^{4}	1	3.2×10^{3}	1	3.1×10^{3}	1	2.06×10^{4}	3	4.1×10^{2}	1	1.04×10^{4}	3
10^{-5}	6.62×10^{3}	2	3.2×10^{2}	0	3.1×10^{2}	0	2.06×10^{3}	2	4.1×10^{1}	1	1.04×10^{3}	2
10^{-6}	6.62×10^{2}	0	3.2×10^{1}	0	3.1×10^{1}	1	2.06×10^{2}	1	4.1×10^{0}	0	1.04×10^{2}	2
Total mortality:		9		3		6		20		8		18

* Four embryos inoculated with each dilution; S = smooth variant; R = rough variant; CG = cut-glass variant.

creased. For this reason, it would be of interest to observe such embryos upon prolonged incubation, exceeding six days, in order to determine the final result of the embryo-invading organism interaction. Of further interest would be the determination of the virulence and antigenic and colonial stability of the organisms in a series of subsequent chicken embryo passages.

With the exception of 1 strain, the variant type inoculated predominated on recovery from embryos. Four variant strains were recovered only in the type inoculated. In the case of five other strains, there was a slight degree of dissociation to mucoid type, and with one strain some dissociation to cut-glass type. Since the latter dissociations were of low order, and in no instance complete smooth to rough or rough to smooth type changes, it is unlikely that they appreciably influenced the final results. However, it would be of interest to evaluate the infectivity of selected mucoid and cut-glass variants for the chicken embryo.

Effect of Cell Numbers Inoculated.—Viable cell counts were calculated from suitable plate counts and revealed an apparent lack of correlation between the number of viable cells inoculated and embryo death rate (table 3A and B.) In some instances, the mortality was higher with a smaller number of viable cells than with a larger number. In no instance was there a clearly increased mortality with increased dosage. It may be possible that a random selection and growth of predominantly virulent organisms occurred from certain serial dilutions of the variant cultures and not from other dilutions upon propagation *in vivo*. Also to be considered is the fact that allantoic fluid is an excellent medium for the propagation of *V. fetus*.[2] This may have permitted the growth of relatively small numbers of viable organisms to reach the infective dose for chicken embryos in a short time.

After standardizing the different variants for embryo inoculation, it was found that the number of viable organisms, in suspensions of the same optical density, varied greatly. Plate counts varied from 4.1×10^6 with the smooth variant of v strain to 1.04×10^9 with the rough variant of 53-D strain. However, increased viable counts did not result in increased death rates.

SUMMARY

Smooth and rough colonial variants of *Vibrio fetus* strains of bovine, ovine, and human origin, and one bovine "saprophytic" strain, were infective for chicken embryos. With one exception, smooth variants were more virulent than rough variants. The bovine saprophytic Vibrio strain demonstrated a relatively high degree of infectivity as compared with *V. fetus* strains. The highest mortality occurred among embryos inoculated with the smooth variant of a strain of human origin. *Vibrio fetus* was recovered from both live and dead embryos; however, lesions were found only in those which died. The colonial variants recovered from infected embryos were predominantly the same as those inoculated. There was no correlation between the number of viable cells inoculated and embryo mortality.

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The Influence of Maturity and Sex on the Blood Pressure of the Turkey

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THE BLOOD PRESSURE of the adult male chicken is 15 to 20 per cent higher than that of the female,[8] but chicks under 8 weeks of age do not exhibit this sex difference.[9] The blood pressures of the male and female duck and pigeon do not differ even after maturity.[6] The position of the turkey in this picture is not clear. Kaupp [4] showed that the tom had the higher pressure, but his data are based on 1 male and 1 female. The only other observation on turkey blood pressure appears to be that of Stubel [7] but this, too, is limited to 1 male. Inasmuch as aortic rupture in turkeys may be related to blood pressure,[3] further knowledge of the hemodynamics of this species could be of practical significance.

MATERIALS AND METHODS

The turkeys were a June 1 hatch of New Jersey Buffs, raised by conventional methods. Poults, 6 to 7 weeks old, provided data on immature birds, and toms and hens, 8 to 9 months old, provided data on mature birds. Direct blood pressure measurements were obtained from the carotid artery of unanesthetized birds by methods previously described.[6, 9, 11] In addition, simultaneous indirect measurements were made with cuffs, 1 and 1½ inches wide, on some of the adults as had been done with the chicken [11] and duck,[6] except that a more sensitive and reliable pulse detector * was used.

RESULTS

Blood pressures and heart rates of the turkeys are listed (table 1). There are no significant differences attributable to sex in the poults, apart from the slightly heavier weight of the male. In the adult, the pulse pressure of the male is significantly higher (by 34%) and the heart rate lower (by 22%) than in the female. In the male, systolic pressure is greater and diastolic pressure less than in the female, but these differences are not significant. The male body weight is nearly twice that of the female.

The systolic and pulse pressures of both the male and female adult birds are significantly higher, and the heart rate significantly lower than in the immature birds. Diastolic pressure remained essentially unchanged. There were no significant correlations in the adult between heart rate and

TABLE 1—Effect of Age and Sex on the Blood Pressure and Heart Rate of the Turkey

	No.	Body wt. (kg.)	Systolic blood pressure (mm. Hg)	Diastolic blood pressure (mm. Hg)	Pulse pressure (mm. Hg)	Heart rate (beats/min.)
			IMMATURE (6 to 7 weeks)			
Male	14	1.1*	197	154	43	310
Female	15	1.0	190	146	44	310
Pooled sub-group s.e.	....	± 0.02	± 5.1	± 3.9	± 2.7	± 6.8
Av. and s.e.	29	.....	193 ± 3.6	150 ± 2.8	43 ± 1.9	306 ± 4.8
			MATURE (8 to 9 months)			
Male	15	9.7*†	226†	152	74*†	144*†
Female	15	5.4†	212†	157	55†	185†
Pooled sub-group s.e.	....	± 0.13	± 6.4	± 4.7	± 4.3	± 5.6
Av. and s.e.	30	.....	219 ± 4.5	154 ± 3.3	.....	.....

* Significant difference between sexes. † Significantly different from immature.

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* "Infraton" pickup Model B, Medical-Electronics Development Co., 12 Crampton Lane, Great Neck, Long Island, N. Y.

body weight on the one hand, and systolic or pulse pressures on the other.

The comparison of simultaneous direct and indirect systolic pressures is listed (table 2). All values obtained with the 1-inch cuff are significantly higher than the direct readings. With the 1½-inch cuff, the indirect values are still elevated, but less than half as much as with the 1-inch cuff. The comparison is better in the female, where the 1½-inch readings are only 9 mm. higher than the direct measurements.

DISCUSSION

Indirect measurements of systolic pressures, even those made with the 1½-inch cuff, the largest available at the time, are unsatisfactory. The better agreement between direct and 1½-inch indirect than with the 1-inch indirect suggests that cuffs 1¾ to 2 inches wide should have been used. As pointed out with the chicken [11] and the duck,[6] cuff width appears to be dependent on animal weight, shape, and size. Where the sexes differ greatly in physical measurements, cuffs of different widths may possibly be required for male and female.

With regard to a difference in pressure and heart rate between the sexes, the turkey appears to be more like the chicken [8, 9] than the duck or pigeon.[6] The sex difference in pressure of the turkey, however, lies mainly in the elevated pulse pressure of the tom, whereas, in the rooster, systolic and diastolic pressures were also elevated. The average pressure levels of the adult turkey appear to be generally higher than those we have recorded for the mature chicken, duck, and pigeon. These values agree with Stubel's [7] single mean pressure value of 193, but are somewhat higher than Kaupp's,[4] which were obtained on anesthetized birds.

Neither the increase in pressure of the mature turkey over the poult nor the elevation of pulse pressure of the adult male over the female can be ascribed to heart rate changes alone, although they appear to be inversely related. Wiggers [12] has shown that a decreased heart rate, other factors remaining constant, may raise pulse pressure, but it simultaneously decreases both systolic and diastolic pressures. Furthermore, the heart rate and pulse pressure were not correlated in either sex. While it is not possible, in the absence of cardiac output data, to specifically delineate the mechanism producing the change in blood pressure, the adult male pattern, in particular, closely resembles the changes attributed to reduced distensibility of the arterial system.[12] It is interesting that the aortas of turkeys dying from internal hemorrhages [2, 3, 5] showed atheromatous changes which are generally associated with decreased distensibility and which, in the chicken, are correlated with blood pressure.[10]

The possible role of blood pressure in the rupture of the dissecting aneurysm has been referred to by Gibson and de Gruchy.[3] Burton's theoretical equilibrium diagrams[1] further show how, in the presence of weakened elastic tissue, a slight increase in pressure may cause vessel "blowout." In view of the generally high pressures of the turkey, which reached 280 systolic in a few males, and with fluctuations of 10 to 20 per cent not uncommon, blood pressure may well be a factor in aortic rupture. Perhaps the effect of the significantly higher male pulse pressure, which reached 100 mm. in several birds, should be considered since aortic rupture occurs more commonly in males.[3] This discussion must be tempered, however, by the realization that rupture of the aorta has been reported only in the large Bronze varieties [3] and not in the

TABLE 2—Differences Between Direct and Indirect Measurements of Systolic Blood Pressure (ΔSBP) in the Turkey

	1-inch cuff			1½-inch cuff		
	No.	Δ SBP (mm. Hg)	Body wt. (kg.)	No.	Δ SBP (mm. Hg)	Body wt. (kg.)
Male	7	30	9.3	9	19	9.5
Female	5	39	5.6	6	9	5.5
Pooled subgroup s.e.*	....	± 5.5	± 0.2	....	± 5.2	± 0.2
Av. and s.e.*	12	34 ±4.0	.....	15	15 ± 4.0	.....

* Standard error of mean difference when comparing the indirect-direct differences, and standard error of mean when comparing subgroups or groups.

smaller Buffs from which these data were obtained.

SUMMARY

There are no differences in blood pressure between the sexes in 6- to 7-week-old Jersey Buff poults for which the following averages were established: systolic 193±3.6, diastolic 150±2.8, pulse pressure 43 ± 1.9, and heart rate 306 ± 4.8. Systolic pressure of the 8- to 9-month-old adult is higher (226 in the male) and heart rate lower than in the poult. The pulse pressure of the adult male (75) was significantly higher than that of the female (55), whereas the heart rate of the female (185) was significantly higher than that of the male (144). Blood pressures for turkeys were higher, in general, than those reported previously for chickens, ducks, and pigeons. The adult male pressure pattern appeared to reflect decreased arterial distensibility, such as might be expected from aortic atheromatosis. The elevated pressures, in turn may initiate spontaneous aneurysms and ruptures of the atherosclerotic vessels.

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The Pathology of Aleutian Disease in Mink

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THE DISEASE hereinafter referred to as "Aleutian disease" was apparently seen as early as 1946 by Hartsough.[3] He observed a condition of hepatonephritis in necropsied Aleutian female mink. Since 1946, deaths in Aleutian stock mink from this disease have been observed in all of the mink-raising areas of North America and Scandinavia (Hartsough and Gorham[4]). The first evidence of the disease was a lack of weight, although interest in food continued and an obvious polydipsia appeared. Dark tarry droppings were occasionally seen and often contained undigested food. About 10 to 20 per cent of the affected animals bled from the mouth and were considered anemic. As the disease progressed, anorexia, lethargy, and cachexia became increasingly apparent. The usual result was death, which occurred at any time after a few days up to several months. Imperial Sapphires were found to be particularly susceptible to the malady.[3]

Pathological alterations reported were: gingivitis and oral ulcers which bled easily, enlarged kidneys studded with stellate petechiae, atrophied and pitted kidneys (in some animals), patchy congestion of the spleen, "nutmeg" liver, and occasional hemorrhages (Hartsough and Gorham[4]).

This paper reports the disease as seen on a large mink ranch in Connecticut.

HISTORY

The mink ranch has been in continuous operation since 1931. The first Aleutian mink, along with Imperial Platinum mink, were introduced in 1947.

In July, 1954, there were 8,350 mink, 6,850 of which were carrying Aleutian or Sapphire blood. The remainder consisted of dark-coated mink chiefly of the Pastel strain. Ordinarily, about 1,800 were kept as breeding stock. The ranch consisted of two areas, one fourth of a mile apart, enclosed with mink-proof wire. Sixteen typical mink sheds of various lengths and 12 feet wide held 3,350 mink in one area of the ranch, and 5,000 in the other. Individual mink were confined in a box and screen enclosure approximately 3 feet above the ground.

The diet, carefully balanced to meet newer concepts of mink nutrition, consisted largely of raw meats from other states and Canada. For about three months in 1954, chlortetracycline (30 Gm./ton) was added as a food supplement. Since the amount was difficult to calculate on the basis of wet food, the practice was discontinued.

In 1953, the owner was "optimistic about future prospects"; he considered that year's loss (188 mink) reasonable. The first indications of trouble were sterility and small litters in 1954.

MATERIALS AND METHODS

Mink were anesthetized and later killed by chloroform. Their body temperatures were taken rectally. Blood samples were obtained by cardiac puncture, then citrated or oxalated. A portion of each sample was used to determine the sedimentation rate and the hematocrit level and to prepare smears stained by the Wright method. The blood serum was subjected to the agglutination-lysis test for leptospirosis. In addition to the antigens of *Leptospira canicola*, *Leptospira icterohaemorrhagiae* and *Leptospira pomona* used routinely by this laboratory, the Walter Reed Army Medical Center, Washington, D.C., subjected five serums to the agglutination-lysis test employing all known leptospiral antigens. Urine for dark-field examination was withdrawn from 5 mink which died of Aleutian disease. Ordinarily mink micturate during the death struggle, making urine difficult to obtain.

Infectivity studies included the inoculation of chicken embryos, guinea pigs, weanling hamsters, kittens, weanling mice, mink, and rabbits with kidney, liver, spleen, and occasionally brain and lung tissue suspensions. They were secured aseptically and usually inoculated within two hours. Freezing of the tissues and the use of antibiotics were avoided.

The previously enumerated tissues were triturated in tryptose broth, 10^{-1} weight/volume, and 0.2 ml. of this diluent was used for chicken embryo inoculations. This amount was injected into the yolk sacs of 5- to 7-day-old chicken embryos, and onto the chorioallantoic membranes (c.a.m.) of 9- to 11-day-old chicken embryos. Tissues were harvested four to six days later from the yolk and chorioallantoic sacs, and 11 to 12 days later from those embryos inoculated on the c.a.m. Tissues were similarly inoculated until four passages had been accomplished. Triturated material was inoculated in the amount of 0.5 ml. into day-old chicks according to the method of Hoag *et al.*[5] These birds were exsanguinated on the fourth day of

From Storrs Agricultural Experiment Station, Storrs, Conn.

This study was supported by the Mink Farmers' Research Foundation.

TABLE 1—Gross and Microscopic Findings in Mink Affected with Aleutian Disease

	Gross observations					Histological observations					
	c.n.s. signs	Oral mouth ulcers	Enlarged kidney	Enlarged liver	Enlarged spleen	Arteritis	Encephalitis	Hepatitis	Myocarditis	Nephritis	Pneumonitis
Present	16	8	28	27	25	11	24	34	4	24	19
Absent	24	32	12	13	15	29	6	6	36	16	21
Total	40	40	40	40	40	40	30*	40	40	40	40

* Ten brains not examined.

age and the blood was inoculated into other day-old chicks and on the c.a.m. of 4- to 6-day-old chicken embryos.

For animal inoculation, the tissues were triturated in normal saline solution. Weanling mice and weanling hamsters were inoculated intracerebrally (i.c.) with 0.03 ml. and intraperitoneally (i.p.) with 0.5 ml. Rabbits were given 1.0 ml. intravenously. Young, full-grown guinea pigs, weanling kittens, and full-grown mink were given 1.0 ml. i.p. Unless test animals died, they were held for 30 days, but mink were held for 60 days and were handled as reported for mink submitted for necropsy.

Fletcher's medium was inoculated with kidney and liver tissues. Serums from mink, horses, cows, and rabbits were used in preparing this medium. Blood agar and Sabouraud's agar plates were streaked with these and any other abnormal tissue. The blood agar plates were incubated at 37 C. under 10 per cent CO_2 tension, while other inoculated mediums were incubated at room temperature and at 32 C.

Tissues for histological study were usually fixed in 10 per cent formalin, although Zenker's solution and Bouin's solution were occasionally used. All tissues were embedded in paraffin and routinely stained by Harris' hematoxylin and eosin. Special stains employed were: Brown and Bren's Gram, Congo red for amyloid, Gridley's fungus, Levaditi's silver, Masson's trichrome, periodic acid Schiff, Unna's plasma cell, and Van Gieson's. Tissues were taken from the bladder, brain, duodenum, heart, ileum, jejunum, kidney, liver, lungs, pancreas, spleen, and stomach.

Liver and stomach contents, in five instances, were subjected to toxicological examination.*

Of 73 mink from the same ranch, 40 were studied as indicated above, and 33 were not considered good specimens for such study.

RESULTS

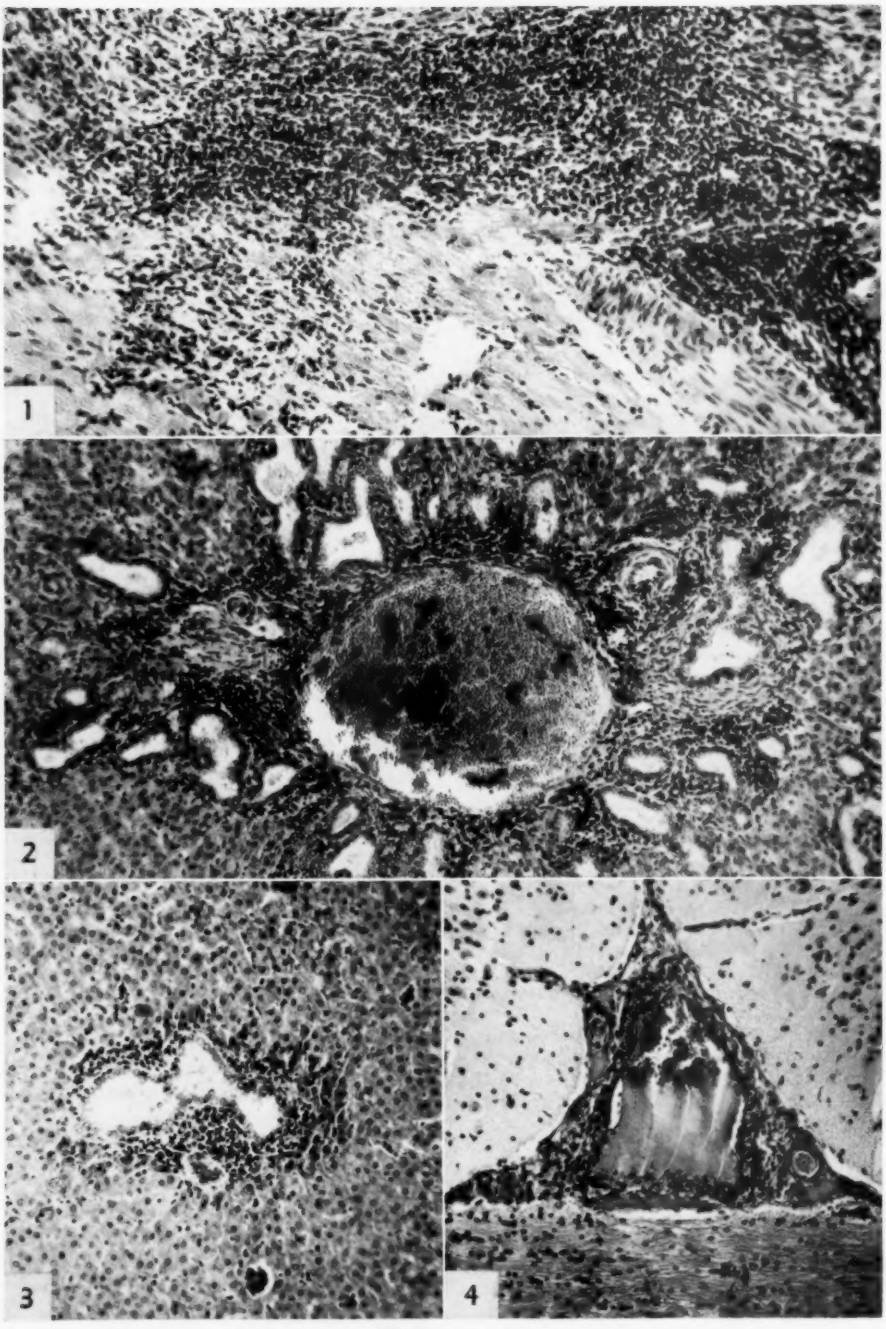
Clinical Observations.—Clinical observations on this herd of mink were made from July 2, 1954, until March 10, 1955. During this time, the disease developed to a point where 5 to 10 mink died each day. However, in the first two months, one or two deaths per day was usual. The first mink submitted were termed "bleeders," as the owner saw blood oozing from the mouth and, in some cases, from the anus. By September of 1954, this sign was minimal. Other signs were anorexia, cachexia, lethargy, and a febrile reaction resulting in temperatures up to 107 F. According to the owner, an important early manifestation was polydipsia, which was particularly noticeable during the season when drinking water was frozen. Affected mink would chew and lick the ice in a frenzied manner. Some degree of central nervous system derangement was present, usually manifested by the affected animal executing a series of body rotations, and later by a comatose attitude. In some individuals, the head and neck were held in a position resembling "screw neck," which is a peculiar behavior pattern seen in Pastels homozygous for brown eyes.[8]

The course of the disease varied considerably. While a few mink died suddenly after a few days of illness, the majority were ill for weeks or even months. Older animals tended to linger for several months. Approximately 90 per cent of the affected animals died within a month, and those which appeared to recover often died at a later date. The owner believed that recovery in definitely established cases was rare.

Deaths totaled 566 by the end of 1954. However, by March 10, 1955, the losses approximated 1,500, since all mink that appeared to be ill were killed.

Gross Pathological Changes.—The mink was usually well developed, but often thin and dehydrated, although the fur was considered good enough for pelting. In the mouth, particularly in the gums and buccal mucosa, shallow ulcers up to 3 mm. in size were occasionally present. These were invariably covered by a diphtheritic membrane and the lesions bled freely when disturbed. Striking changes occurred in the liver, kidney, and spleen. The kidneys were often three times normal size. They were pale orange to yellowish, and frequently

* Dr. Harry Fisher, New Haven Agricultural Experiment Station, New Haven, Conn., performed these examinations.



Figures 1 to 4

All figures are photomicrographs of mink tissue embedded in paraffin and stained with hematoxylin-eosin.

presented a mottled appearance due to countless punctiform whitish areas. Stellate subcapsular hemorrhages were common and the capsule stripped easily. When cut, the architectural distinction of the various component parts was maintained.

The liver was enlarged, up to twice normal size, and presented a mahogany hue. The gallbladder was normal.

Aside from a two- to fivefold increase in size, the spleen was considered normal.

The surface areas, as well as deeper tissue, of the lungs were marked by deep red patches which contrasted with the pale pink of the normal lung (table 1).

Free blood was found in the colon in only five instances.

Histopathological Changes.—In organs showing gross alterations, histological changes were generally marked and extensive (table 1). Only the arterial side of the vascular system was affected, particularly after prolonged illness. When these vascular changes occurred, not a single organ or tissue was spared.

In the liver, the earliest alteration was slight infiltration of the triads by plasmacytes and a few lymphocytes (fig. 3). This might be the earliest lesion of the disease, as it was the only one seen in animals with suspected field cases. Some were in mink only 2 months old. As the condition progressed, bile duct infiltration and dilatation with the omnipresent cellular infiltrates were seen (fig. 2). The impression was that the reaction of the liver was a chronic inflammatory response. Necrosis and fibrosis were always lacking.

In the kidney, the change was primarily an interstitial nephritis. Although chronic interstitial nephritis was usually present, kidneys were seen in which the nephritis was acute, focal, or diffuse in character. The acute type of nephritis was marked by the interstitial infiltration of groups of plasmacytes and lymphocytes (fig. 12). As in the liver, the plasmacytes were present in relatively large numbers. In chronic nephritis the alterations were marked. The cortical interstitial tissue was crowded with plasmacytes, lymphocytes, and histiocytes. Some glomeruli and tubules were destroyed, others were atrophied, and some were represented by hyaline globules (fig. 13 and 14). The tubular epithelial cells often contained large vacuoles which were strongly argentophilic when sections were stained by Levaditi's silver technique. The significance of these vacuoles has not been determined.

The lung consistently presented a picture of alveolar wall thickening due to infiltration of types of round cells (fig 15). This lesion failed to evoke any clinical signs, and it was of no apparent diagnostic value, inasmuch as it commonly occurred in other diseases. This type of lesion has been reported in human nephritic patients.[1]

The spleen maintained its architectural integrity. Thus, a compensatory reaction was the only reasonable explanation for its gross increase in size.

The brain alterations were both proliferative and degenerative. The earliest determinable lesion was a loose, but focal, microgliosis including a few lymphocytes. As the lesion matured, the gliosis became dense to the point of forming nodules. Perivascular cuffs appeared, along with areas of demyelination, satellitosis, and neuronophagia. At all times, the focal nature of the lesions persisted, so that a disseminated encephalitis was never seen. A spotty nonpurulent leptomeningitis often accompanied the other brain changes (fig. 4-7).

The involvement of the arterial side of the vascular system was an outstanding lesion (table 1). The earliest manifestation

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Fig. 1—Myocardium from a 10-month-old Sapphire female mink showing massive lymphocytic infiltration. x 90.

Fig. 2—Section from the liver of a 12-month-old Sapphire male mink. The portal triad shows marked bile duct dilatation and proliferation, with infiltration by lymphocytes and plasma cells. x 90.

Fig. 3—Section from the liver of an 18-month-old Sapphire female mink showing a portal triad slightly infiltrated by plasma cells. x 10.

Fig. 4—A cross-section of the inferior saggital sinus from the brain of an 8-month-old Sapphire female mink showing a slight perisinusoidal infiltration. x 90.

of this pathological process was the appearance of histiocytic aggregates in the adventitia (fig. 9). The nodule increased in extent to form a perivascular cuff, with the addition of some lymphocytes (fig. 10). The lesion encroached upon the media, followed by hyalinization of media and adventitia (fig. 10). The intima was not directly affected but underwent hypoplasia which was probably due to the presence of the hyalin. A reduced patency was maintained although the anatomical components of the artery had been destroyed. Eventually, a hyalin plug filled the lumen, resulting in a mass of hyalin loosely surrounded by lymphocytes. Veins were not involved and it was not uncommon to see an affected artery next to an unaffected vein (fig. 16). If the intensity and extent of the other lesions were accepted criteria of their age, the vascular changes appeared late in the course of the disease. No organ or tissue was spared. For example, the stomach or urinary bladder might not be affected in the sense of gastritis or urocystitis, yet might present vascular lesions (fig. 8 and 11).

Pathogenesis.—Specimens from 2-month-old mink which had recently become naturally infected with Aleutian disease, as well as from mink with experimentally induced cases, suggested that the earliest lesions appeared in the liver, followed shortly by changes in the kidney. Lesions were present in these two organs throughout the course of the disease (table 1). In those instances where arteritis occurred, such changes were seen simultaneously in many organs (fig. 1).

Bacteriological and Mycological Findings.—No organisms were recovered consistently. *Erysipelothrix rhusiopathiae* was recovered from oral ulcers of 2 mink.[2] This isolation was never duplicated. Occasionally a *beta*-hemolytic Streptococcus was isolated. The inconstant recovery of such organisms, in addition to the failure of Aleutian disease to respond to antibiotics, led the authors to consider them as secondary invaders.

TABLE 2—Laboratory Diagnosis of Mink Diseases in Connecticut from Jan. 1, 1938, to Jan. 1, 1956

Disease	Number of cases of each disease	Number of mink affected
Abscess	1	1
Aleutian disease	34	119*
Botulism	3	3
Chastek's paralysis	1	1
Coccidiosis	2	2
Cystitis (Uro-)	3	3
Distemper, canine	9	26
Encephalitis	3	3
Gastritis, ulcerative	1	1
Injury	2	3
Lead poisoning	2	3
Mercury poisoning	1	2
Mycobacteriosis	8	12
Nephritis	2	5
Rickets	1	1
Steatitis (yellow fat)	11	18
Undiagnosed	4	16

* All diagnosed since July 1, 1954.

The leptospiral and mycological mediums always remained without growth.

Virological Findings.—No conclusive virological findings resulted from this study. Embryonic deaths and lesions on the c.a.m. were encountered in only isolated instances on initial inoculations. Lesions were considered traumatic in origin and did not appear during serial passage.

Animal Inoculation Studies.—No animal other than the mink has proved susceptible to Aleutian disease. Inoculated laboratory animals invariably remained healthy and were considered normal when examined grossly and histologically. However, 2 mink of the Aleutian strain showed signs of anorexia and lethargy 30 days after a 1-ml. (i.p.) inoculation of pooled liver and kidney in a 1:10 dilution in saline solution. Thirty days after the first sign of illness, the animals were killed and their pooled livers and kidneys were inoculated i.p. into an apparently healthy Aleutian mink. This animal followed the same course of illness and was killed 60 days after inoculation. In all 3 animals, slight changes of the type

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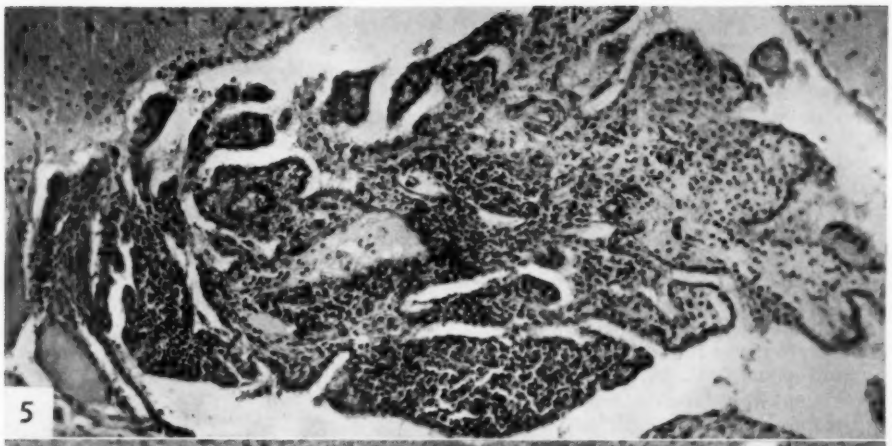
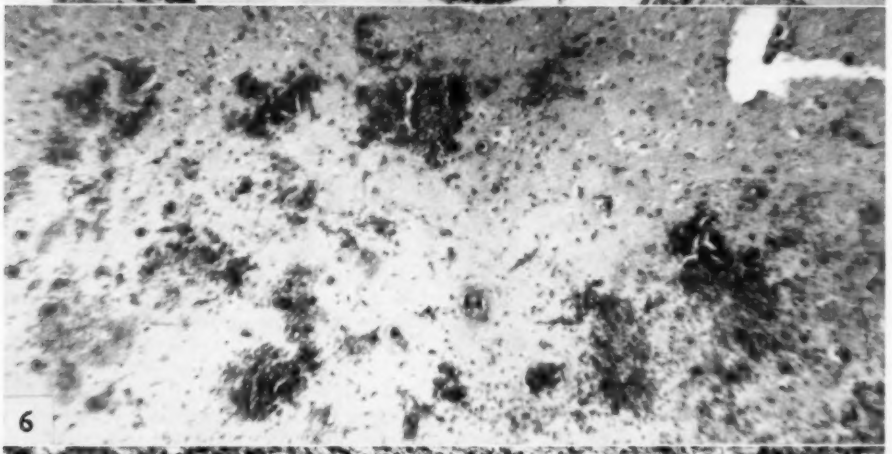
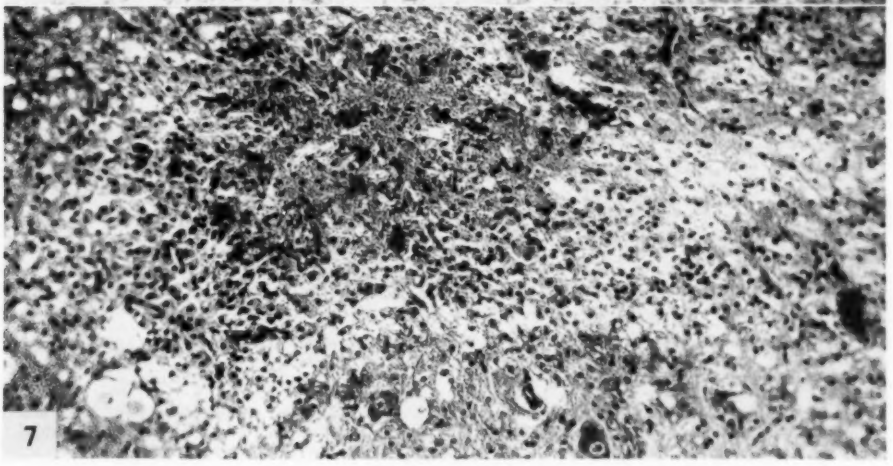
Fig. 5—Choroid plexus of the lateral ventricle from the brain of an 8-month-old Aleutian female mink showing extensive lymphocytic infiltration. x 90.

Fig. 6—Thalamus from the brain of a 3-month-old Sapphire female mink, with multiple hemorrhages in an area of encephalomalacia. x 90.

Fig. 7—The nucleus ruber from the brain of an 8-month-old mink showing a mass of cellular debris and pyknotic neurons surrounded by slight lymphocytic infiltration. Recognizable neurons are present in extreme lower border. x 105.







Figures 5 to 7

All figures are photomicrographs of mink tissue embedded in paraffin and stained with hematoxylin-eosin.

seen in field cases were seen in the liver and kidneys. A few brown mink and ferrets, inoculated at another laboratory,* failed to become ill. The only Aleutian mink available for study were those from the ranch involved and they may have been affected when inoculated.

Serological Examinations. — The blood serums of the mink, as well as of the rats and cats trapped at the ranch, were negative for antibodies to any of the known leptospiral antigens.

Toxicological Findings.—Poisonous substances were never found in contents of the liver and stomach.

Treatment and Prophylaxis.—Treatment and prophylaxis were ineffective. Sulfamethazine given in the drinking water (1 oz./gal.) resulted in mink often refusing to drink. Intraperitoneal injections of the sodium form, up to 5 gr. per pound of body weight, failed to produce beneficial results. Penicillin and dihydrostreptomycin, alone and combined, were given in amounts up to lethal doses with no apparent benefit. Chlortetracycline, oxytetracycline, and tetracycline hydrochloride were employed as therapeutic agents by injections, and as prophylactics by feeding (40 Gm./ton) in wet feed. These antibiotics failed to halt the disease.

When *Ery. rhusiopathiae* was recovered from the first cases, penicillin was used therapeutically and a commercial erysipelas vaccine (Duovax†) was used prophylactically, but without effect. As the disease progressed, a diagnosis of leptospirosis was considered. A commercial bivalent *L. canicola* and *L. pomona* vaccine was injected into 600 mink with no appreciable results.

At this point, the owner made a vaccine by emulsifying kidney, liver, and spleen in 10 parts of water, and adding 0.5 per cent commercial formaldehyde by volume. The mixture proved to be bacteriologically sterile. Over 1,000 mink were inoculated subcutaneously with 1 ml. of the supernatant fluid. Although there was no immediate reaction, by the end of six months almost all these mink were dead of Aleutian disease. (It was the owner's opinion that he spread the disease by inoculating and handling, or by the vaccine itself.)

DISCUSSION

Aleutian disease was observed to occur as a chronic systemic disease with histological lesions of the type associated with leptospirosis. However, Levaditi's silver technique failed to reveal spirochetes in affected tissue, except in 3 mink. This fact, plus questionable dark-field urine examinations, the failure of various inoculated laboratory animals to develop leptospirosis, the inability of serological tests to detect corresponding antibodies in affected mink and captured rats, and the lack of protection from leptospiral vaccine, precluded a diagnosis of leptospirosis.

The course of the disease suggested an etiological agent of an infectious character. Most kits in a litter died of the acute form, whereas the dam became ill but usually remained alive for months. At first, the disease appeared to be confined to a few pens but within several months it was seen in all parts of the ranch. In spite of this obvious picture of contagion, no pathogenic agent was isolated.

The possibility that some ingredient of the ration was at fault was considered but

* Farm and Home Division of the American Cyanamid Co., New York, N.Y.

† Douvax, product of American Cyanamid Co., New York, N.Y.

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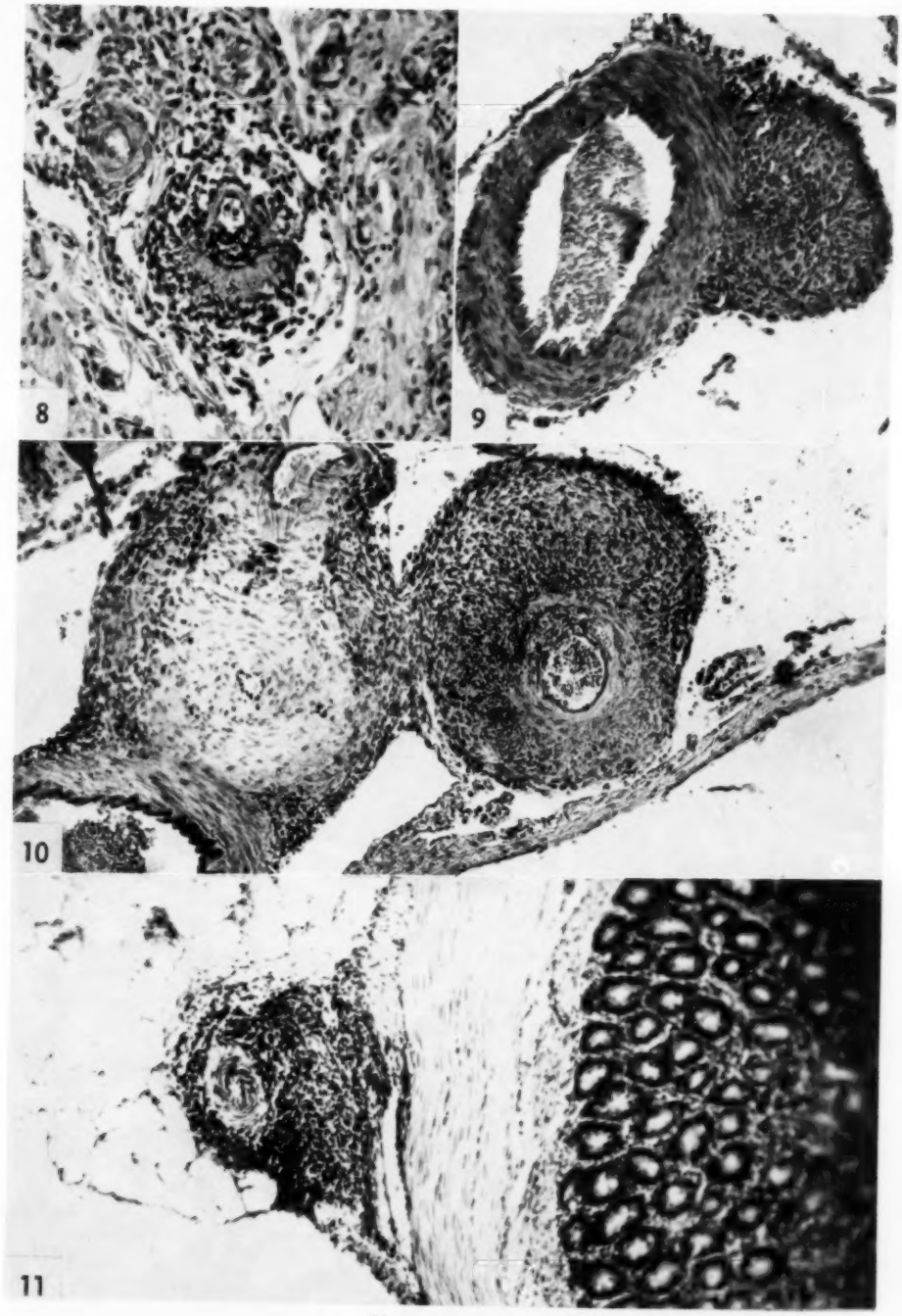
Fig. 8—Section from the muscle of the stomach of the mink shown in figure 7. There is slight lymphocytic periarteriolitis and hyalinization of the lower half of the arteriolar wall. x 98.

Fig. 9—Cross-section of a branch of the dorsal inferior cerebellar artery in the region of the fourth ventricle from a 5-month-old Sapphire male mink showing early vascular change consisting of a histiocytic nodule in the periadventitial aspect. x 90.

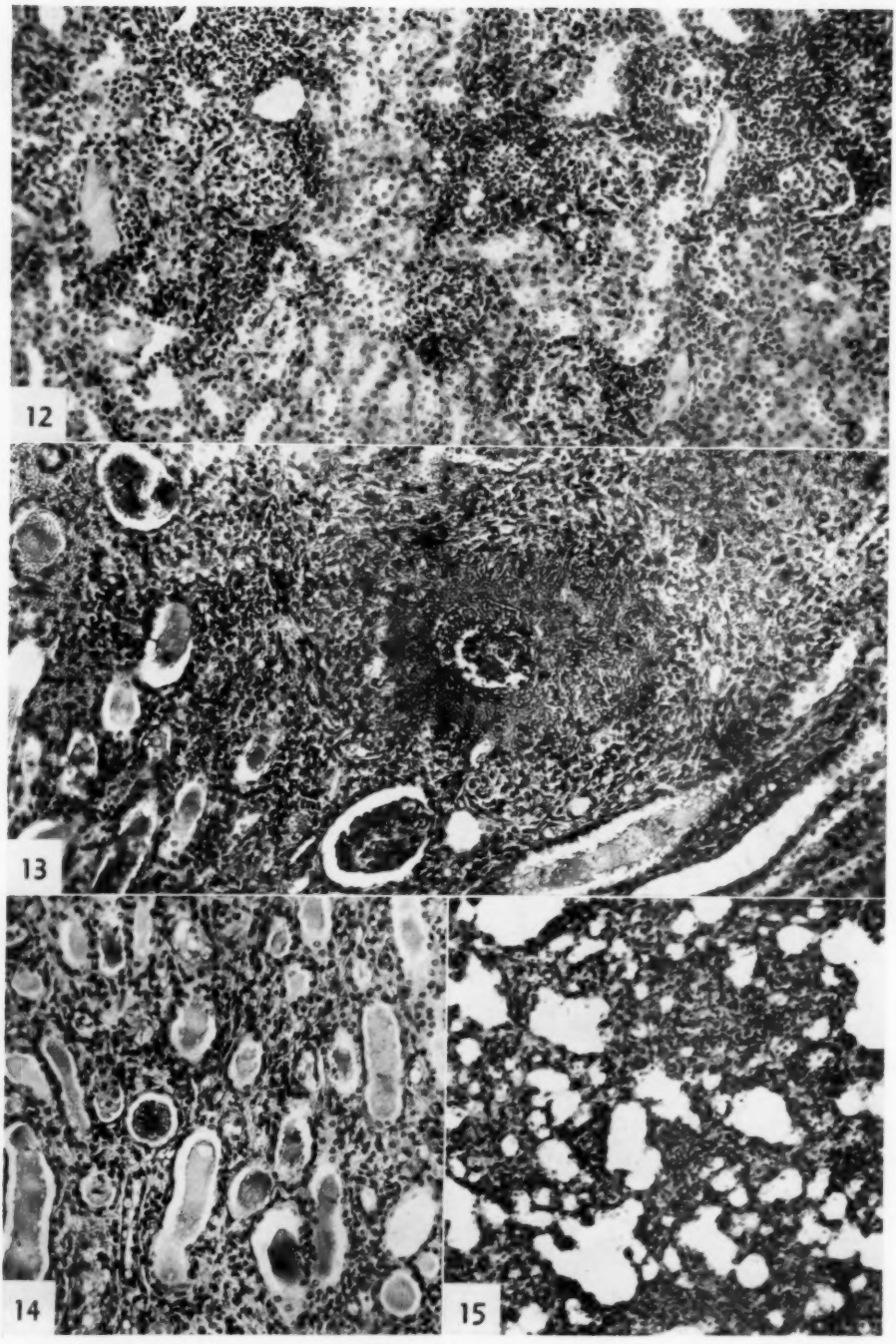
Fig. 10—Cross-sections of twin branches of the dorsal inferior cerebellar artery from an 8-month-old Sapphire female mink. The artery to the right shows infiltration by histiocytes, the one to the left shows chiefly mural hypertrophy, a polyarteritis. x 90.

Fig. 11—Cross-section of a branch of the mesenteric artery from a 5-month-old Aleutian female mink showing a fairly compact cuff of lymphocytes about the artery. The muscular coat and mucosa of the jejunum are to the right. x 82.



Figures 8 to 11

All figures are photomicrographs of mink tissue embedded in paraffin and stained with hematoxylin-eosin.



Figures 12 to 15
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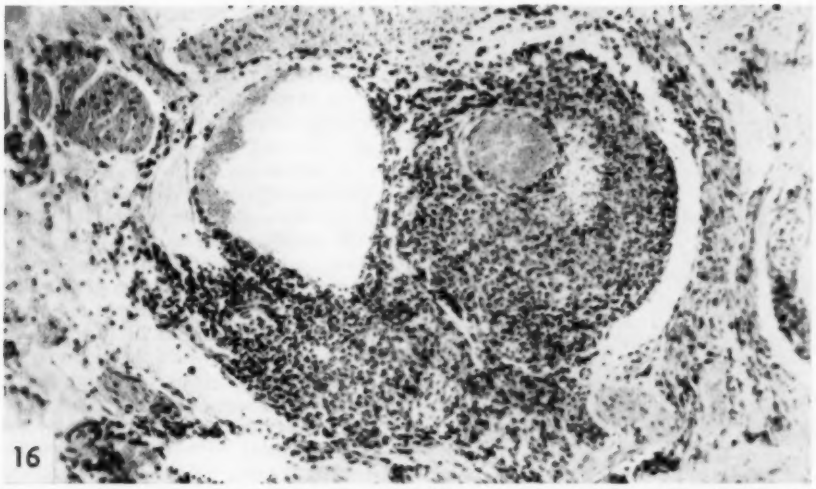

Fig. 16—Section from the wall of the urinary bladder of a 5-month-old Sapphire female mink. The artery shows polyarteritis while the vein to the left is apparently unaffected. H & E; x 90.

could not be substantiated. Other small mink ranches were feeding the same ration while another moderately large ranch was feeding a portion of the ration supplied to the affected ranch, and the food materials were mixed on the grounds of the affected ranch and transported by truck. Since these other ranches were not affected, it was concluded that the ration was not at fault and that if an etiological agent existed in the food, it did not gain entrance to the body *per os*.

Aleutian disease in mink can be diagnosed by gross observation. The chronic nature of the disease and the high incidence among Aleutian-Sapphire strains, associated with anorexia, polydipsia, and a high mortality, are most suggestive. The macroscopic lesions, consisting of enlarged, pale, mottled kidneys with enlarged liver and spleen, are characteristic. Any doubts can be eliminated by histological examination. The marked hepatonephritis and less prominent arteritis are pathognomonic.

However, other diseases of mink that pose diagnostic problems have been encountered in Connecticut (table 2). Canine distemper of mink is an acute disease with high morbidity and mortality. Gross observation reveals a mucopurulent rhinitis and conjunctivitis, swollen and cracked foot pads (snowshoe feet) and, frequently, a swollen anus. The pathognomonic inclusion bodies[7] are usually common in the transitional epithelium of the lower urinary tract.

Mycobacteriosis in mink, caused by an acid-fast bacillus most closely resembling

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Fig. 12—Section of a kidney of a 6-month-old Sapphire female mink. Infiltrating lymphocytes and plasma cells occupy a large portion of the section. This is acute diffuse interstitial nephritis. x 90.

Fig. 13—Section of a kidney, from the mink shown in figure 7, showing a hyalinized artery in the center. Eosinophilic pigmented casts are seen in dilated tubules amid a background of cellular infiltrates. At bottom center is an atrophied glomerulus. x 98.

Fig. 14—Section from the medulla of the same kidney as shown in figure 13. The collecting tubules contain prominent casts. x 98.

Fig. 15—Section from the lung of a 6-month-old Sapphire female mink. Small round cells crowd the interstitial tissue and compress the alveoli. x 90.

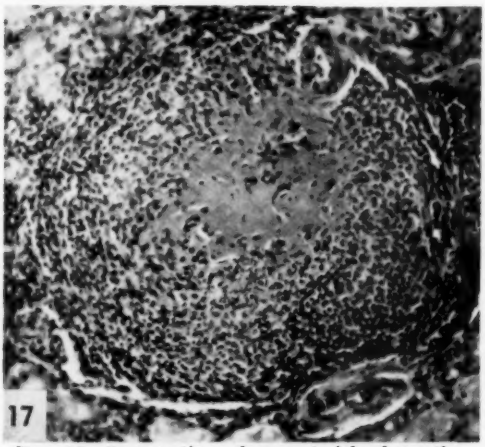

Fig. 17—Cross-section of an arteriole from the kidney of an 8-month-old Sapphire female mink. Arteritis and subsequent hyalinization has resulted in complete occlusion of the vessel. H & E; x 88.

the avian type, has been seen.[6] This disease also attacked Aleuthian-Sapphires almost exclusively. It is a chronic disease and causes a typical "potbelly" due to an enlarged spleen, which is easily palpable. The gross examination reveals nodules scattered throughout the viscera. Histological preparations of these nodules stained by acid-fast methods disclose masses of epithelioid cells which contain many acid-fast rods.

A condition which resembled Aleutian disease in some respects, was fat disease or lipidosis. In an advanced case, the friable orange-hued liver left no question as to the identity of the disease. However, it appears that early cases of lipidosis often go undiagnosed or are incorrectly considered to be Aleutian disease. In such animals, anorexia, polydipsia, and a vague state of illness exists. Animals may die suddenly, particularly following handling. This sudden death is usually the result of rupture of the liver, probably caused by vigorous resistance to being handled. Upon histological examination, there is no cellular infiltrative process. Only fatty infiltration of the liver, and at times of the kidney tubules, is found. Furthermore, correction of the diet and therapy with lipotropic factors often results in distinct improvement and recovery of affected animals.

SUMMARY

Aleutian disease in mink occurred chiefly in Aleutian-Sapphire mutants. It appeared to be a chronic progressive disease of high mortality characterized by anorexia, lethargy, loss of weight, polydipsia, and hemorrhages.

The kidneys were enlarged, pale yellow, and mottled; the liver was slightly, and the spleen greatly, enlarged.

Histological alterations were characterized, in the periportal areas of the liver, by marked plasma cell infiltration with bile duct proliferation, and in the interstitium of the kidney by hyalinized glomeruli, tubular casts, and sclerosis. A polyarteritis appeared in some animals and involved smaller arteries regardless of body location.

No bacterial or viral agents were consistently isolated. *Erysipelas rhusiopathiae* was isolated from only one group. Leptospirosis was suspected but could not be confirmed by bacteriological, serological, or histological studies, or by animal inoculations.

Vaccination of groups of mink for swine erysipelas, for leptospirosis, and with an autogenous vaccine failed to produce protection. No benefit was obtained by the use of sulfonamides and antibiotics.

Aleutian disease can be differentiated from canine distemper, fat disease, and mycobacteriasis by histological examination.

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Egg-Adaptation of a Strain of Distemper Virus Isolated from a Raccoon

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THIS REPORT deals with a strain of canine distemper virus which was isolated from a wild Wisconsin raccoon and maintained in the virus repository of the Department of Veterinary Science, University of Wisconsin, as raccoon 9 strain, distemper virus (R_9–DV).

PROCEDURE

A sick raccoon was submitted to the Department of Veterinary Science in 1954 for diagnosis. The animal evinced typical signs of distemper, *i.e.*, weakness, sniffling, and mucopurulent discharge from the eyes and nose. It was killed and tissues were removed for further study. On necropsy, specific gross lesions were not observed but, microscopically, large numbers of typical distemper inclusion bodies were seen in epithelial cells of the lung, trachea, and urinary bladder. Duplicate sections were stained with hematoxylin and eosin and with Shorr's[7] S_2 stain, as modified by Page and Green.[8]

Samples of spleen and lung tissue were mixed and ground in a tissue grinder. A 1:10 dilution of the tissue was made in sterile broth containing 1,000 units of penicillin and 10,000 μg. of dihydrostreptomycin per milliliter. There was no growth of bacteria in culture mediums inoculated with the mixture. The suspension (1 ml.) was injected into both a cat and a ferret. The cat was also fed several grams of the raccoon tissues, and remained apparently well, but the ferret became sick ten days postinjection, with signs typical of distemper, including lethargy and conjunctivitis associated with a copious mucopurulent discharge from the eyes. At 18 days postinjection, the ferret began to show evidence of improvement and was killed to provide tissues for additional work.

A 10 per cent suspension of the lung and spleen homogenate from the ferret was made in antibiotic broth and inoculated onto the chorioallantoic membranes of 6, 7-day embryonating chicken eggs. The Brandly technique,[1] in which the inoculum is injected between the inner shell membrane and the chorioallantois immediately beneath the natural air cell of the egg, was used. The chorioallantoic membranes of these eggs were harvested five days postinoculation. A 10 per cent suspension of the homogenate of these membranes was inoculated onto the chorioallantoic membranes of another 6, 7-day embryonating chicken eggs. They were reincubated for five days, as in the original passage. The procedure was repeated without variation through five blind passages. Several egg-passage lines were initiated from the fifth passage membranes.

Changes which were apparently the result of viral action began to appear on the chorioallantoic membranes in passage 6. They were typical of the lesions produced by distemper virus, *i.e.*, rough, thickened, wrinkled, and pocklike opacities of varying degree, accompanied by edema of the membrane. The lesions did not appear regularly; at times, entire groups of eggs had apparently normal membranes following inoculation and reinoculation, but changes would reappear on subsequent egg passages.

On the nineteenth passage, one line of the virus became established and regularly continued to produce well-defined lesions on the chorioallantois. The other lines eventually lost all such activity and were discontinued. Several lines were again initiated from the well-established nineteenth passage membranes to assure preservation of the strain.

RESULTS AND DISCUSSION

A 10 per cent suspension of a twenty-second passage chorioallantoic membrane was injected into 2 susceptible ferrets. The ferrets remained well and later withstood challenge by Green's distemperoid virus, a strain known to be highly virulent for ferrets. A control ferret succumbed to the same challenge. Three mink were similarly injected with membranes of the twenty-ninth passage of R_9–DV. They remained well and were resistant to challenge by a virulent field strain of distemper virus, which was obtained from a mink ranch on which a serious outbreak occurred.

Serum from these mink was assayed for virus-neutralizing antibody against distemper virus strain FxNO in embryonating eggs. Serum dilutions of 1:800 neutralized a 1:100 dilution of the virus. The $i.d._{50}$ titer of the FxNO strain was not known, but was ordinarily about 1:1,000.

The R_9 strain has been maintained in

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ferrets as well as in embryonating eggs. It has always produced frank, clinical distemper in ferrets, even after storage at —17 C. for approximately one year. Ferrets have occasionally recovered from infection with strain R_9, which is unusual for the species, although not totally unprecedented. When regularly cultivated on chorioallantoic membranes, strain R_9 produces well-defined lesions over 3 or 4 sq. cm. of the membrane. By passage in eggs, viable virus has been demonstrated, without lyophilization, in those membranes stored at —17 C. for seven months.

The number of strains of distemper virus adapted to the chorioallantoic membrane is not large. The writers and others working at the University of Wisconsin failed to adapt several strains of known distemper virus to eggs, and Plummer [6] reported that a strain of the virus, obtained by him from ferrets and cultivated in eggs, failed to produce changes on the chorioallantois after nine passages. Haig [4] adapted Green's [3] distemperoid virus to the chorioallantois of chicken eggs and produced the first "avianized" distemper vaccine in 1948 and 1949. Cabasso and Cox [2] adapted a strain (Lederle) obtained from ferrets in 1949, and West [8] adapted a fox strain (FxNO) to egg membranes in 1952. Other strains of egg-adapted distemper virus may be in existence, but the number is not great.

SUMMARY

A virus isolated from a wild raccoon has been identified as distemper virus and was carried in serial passage in ferrets for three years. It was adapted to the chorioallantoic membranes of embryonating chicken eggs, where it has been propagated for over 40 passages. The egg-adapted virus is immunogenic, but avirulent, for ferrets and mink.

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Detection of Pulmonary Tuberculosis in Laboratory Monkeys by Chest Radiography

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A RECENT publication [1] indicated that chest radiograph studies are of no value in the antemortem diagnosis of tuberculosis in monkeys. In an earlier publication,[5] lesions were reported "difficult to see even when plentifully spread throughout the lungs." We present evidence to the contrary and also suggest, from our experience, that the use of radiography in the laboratory animal colony, in addition to the palpebral tuberculin test, might be a valuable aid in detecting pulmonary tuberculosis. Our findings are consistent with those of Fox [2] who found that the markings on radiographs prepared from excised monkey lungs corresponded to the position of tuberculous lesions seen on careful gross dissection.

Kennard and Willner [5] concluded, after several years of radiography of laboratory monkeys, that the procedure was too time-consuming and expensive for practical use in a large colony. In their experience, the disease was frequently widely spread throughout the abdominal organs before it reached the lungs. The absence of a fibrous wall or calcification made the lesions difficult to see on radiograph film, and clinical inspection served as well for the detection of tuberculosis in monkeys. They stated that diagnosis during life must be made both by clinical inspection and by the use of tuberculin tests. They also pointed out that their greatest source of inaccuracy was a negative reaction to the tuberculin test when, in actuality, overwhelming infection was present.

Benson, Fremming, and Young [1] recently reported their inability to establish positive antemortem diagnoses of tuberculosis by roentgenography in a colony of 303 monkeys, although 17 reacted positively to the intrapalpebral injection of 10 mg. of Koch's Old Tuberculin. At necropsy the diagnosis was confirmed in 16 of the 17 animals. At additional necropsies, tuberculosis was found in 2 animals which had shown a negative reaction to the tuberculin test. Their report does not describe the degree of pulmonary involvement in the infected animals in which tuberculosis was not detected by radiography nor does it specify the interval of time between the tuberculin test and the necropsies on the tuberculous animals in which the disease was not detected by the tuberculin test.

Although the tuberculin test has a high degree of accuracy in screening for tuberculosis in monkeys,[4] Habel [3] determined that 35 per cent of the monkeys which showed a positive reaction to the intracutaneous tuberculin test (5 mg. of Koch's Old Tuberculin) died within six weeks after exhibiting the first positive reaction, and 85 per cent died within 16 weeks. The monkeys used by Habel were tuberculin-negative three months prior to the first positive test. This illustrates the rapidity with which tuberculosis in monkeys may proceed to its fatal termination. Macfadyen [6] fed tuberculous sputum to 8 monkeys, all of which developed tuberculosis and died after four to nine weeks. Thus, it is conceivable that previously healthy, tuberculin-negative animals could become severely diseased and anergic between routine tuberculin tests yet might continue to give a negative tuberculin reaction. Some additional test should be available for the detection of tuberculous, but tuberculin-negative, animals.

MATERIALS AND METHODS

The tuberculin test and chest radiograph were used in conjunction as a routine screening procedure.

Monkeys in two separate colonies were studied. Colony I (University of Illinois) averaged 25 animals which were kept isolated in a locked room. In order to minimize exposure of the colony to tuberculosis, newly acquired monkeys were kept isolated in a separate locked room for three months. All personnel wore face masks, and access to the animals was strictly limited to authorized persons. Palpebral tuberculin tests and chest radiographs were made every two to three months, followed by euthanasia of suspicious animals.

Colony II (Presbyterian Hospital), consisting of 17 animals divided between two rooms, was not kept under isolation. New animals were not tested as they were acquired, but the entire colony was tested immediately after the necropsy of 1 sickly animal revealed probable active disseminated tuberculosis. Chest radiographs were again taken of all, and tuberculin tests were made on some of the animals after some developed tuberculosis in this room three months later.

The tuberculin tests initially used for colony I consisted of 0.1 ml. of Koch's Old Tuberculin, in a dilution of 1:10, injected intradermally into the right upper eyelid. The tuberculin was diluted with sterile water immediately before use. Subsequently, undiluted tuberculin was used. Colony

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TABLE 1—Radiographic Chest Findings in an Isolated Monkey Colony (I)

Monkey (No.)	Months kept before test	Test results		Disposition	Focus of lesions at necropsy	
		Tuberculin *	Radiograph		Lungs	Mesentery
58	10	—	—	....	....	....
58	12	—	+	Euthanasia	+	— †
77	5	—	—	....	....	....
77	7	Suspicious	—	....	....	....
77	7 (+ 2 days)	—	....	....	....	....
77	8	....	+	Euthanasia	+	+
78	5	—	—	....	....	....
78	7	Suspicious	—	....	....	....
78	7 (+ 2 days)	—	....	....	....	....
78	8	....	+	Euthanasia	+	+
82	2	....	Suspicious	Euthanasia	—	—
Twenty-three other monkeys	Tested every three months for an average of 24 months	— for 18 months; — for next six months ‡	All —	All still alive	....	....

* Dilution of 1:10 used unless otherwise specified. † Spleen contained tuberculous abscesses.
‡ Undiluted tuberculin.

II was initially tested with 0.1 ml. of tuberculin in a dilution of 1:1,000, injected intradermally in the epigastric area. Subsequent tests on 2 animals of this colony were made, using 0.1 ml. of old tuberculin, in a dilution of 1:10, and 0.1 ml. undiluted, injected subcutaneously into the upper eyelid.

Radiographs were taken at 100 ma., 48 k.v.p., at a 36-inch target distance, with an exposure time of one tenth of a second. Cassettes with double intensifying screens were used. A Machlett Dynamax 25 x-ray tube was used with no added filter. Inherent filtration was equivalent to 0.5 mm. Al.

A wall-mounted cassette holder was used. The monkeys were held in an upright position, with the shoulders extended laterally and rotated forward for a posterior-anterior view. Films were sight-developed.

RESULTS

In colony I (table 1), chest radiographs (fig. 1–3) revealed pulmonary lesions in 3 of 27 animals. The tuberculin test (0.1 ml. of Old Tuberculin in a dilution of 1:10) was negative in animal 58 when performed two days before the radiographs were taken. One month prior to taking radiographs which showed lesions, monkeys 77 and 78 had exhibited negative reactions to the tuberculin test and their chest radiographs showed no lesions. Necropsies revealed extensive pulmonic involvement in all 3 animals.

The diagnosis of tuberculosis was made on the basis of the gross appearance of the

TABLE 2—Tuberculosis Infection in Colony II

Monkey (No.)	Test results, 3-3-56		Test results, 6-14-56		Disposition	Focus of lesions at necropsy	
	Tuberculin 0.1 ml., 1:1,000	Chest radiograph	Tuberculin 0.1 ml., 1:10	Chest radiograph		Lungs	Mesentery
2	—	—	....	+	Euthanasia	+	—
3	—	—	....	+	Euthanasia	+	—
12	—	—	....	+	Euthanasia	+	—
4	—	—	....	+	Euthanasia	+	+
6	—	—	....	+	Euthanasia	+	+
7	—	—	+	—	Treated with streytomycin	—	+
10	—	—	+	—	Euthanasia after 6 weeks Treated with streytomycin	—	+
13	—	—	....	—	Euthanasia after 6 weeks Treated with streytomycin	—	+
14	—	—	....	....	Euthanasia after 3 weeks Died of tuberculosis	+	+

These animals (except 15, not listed) were kept for 16 months with no evidence of disease. Because of malnutrition, thought to be due to known gastrointestinal parasites, monkey 15 was killed on Feb. 27, 1956. When probable active tuberculosis was found at necropsy, the remaining animals were tested early March 3, as listed above. They were tested again June 14, after the death of monkey 14, in which active tuberculosis was found at necropsy.

numerous large caseous abscesses present in the lungs. No cultures or other laboratory tests were deemed necessary to confirm this diagnosis. The 23 monkeys remaining in colony I did not develop tuberculosis. They were subsequently tuberculin tested and radiographed every three months for 24 months. Another animal (82) was considered suspicious when radiographed (fig. 4) but was proved nontuberculous at necropsy and by culture.

In colony II, the initial tuberculous animal (15, not shown in table 2) had been purchased 13 months prior to its death; whether it was infected when purchased, or acquired the infection later from a human contact, is not known.

The other 9 animals (table 2) in the room at that time (March 3, 1956) were negative to tuberculin tests and their radiographs showed no evidence of tuberculosis. None of them gave any evidence of ill health.

Thirteen weeks later, on June 14, 1956, 1 animal (monkey 14) died, and fulminating, disseminated tuberculosis was found at necropsy. Chest radiographs of the remaining 8 animals revealed severe pulmonic involvement in 5. These were killed and the diagnosis confirmed at necropsy. Streptomycin was administered (400 mg. i.m.) twice weekly to the 3 for which radiographs showed no evidence of tuberculosis until they were killed. One, which was killed after three weeks, showed tuberculous mesenteric adenitis, but no pulmonary lesions. The other 2 reacted strongly to palpebral tuberculin tests. When killed, six weeks after their last radiograph, tuberculous mesenteric adenitis but no pulmonary lesions were found at necropsy.

In colony II, the diagnosis of tuberculosis was confirmed in all instances by smears in which acid-fast bacilli were found.

A photograph of tuberculous abscesses in the spleen of a monkey is shown (fig. 5).

DISCUSSION

The spread of tuberculosis through colony I ceased as soon as the infected animals were removed. Had we depended upon the tuberculin test alone, at least 1, and possibly more, of the infected animals might not have been detected in time to prevent con-

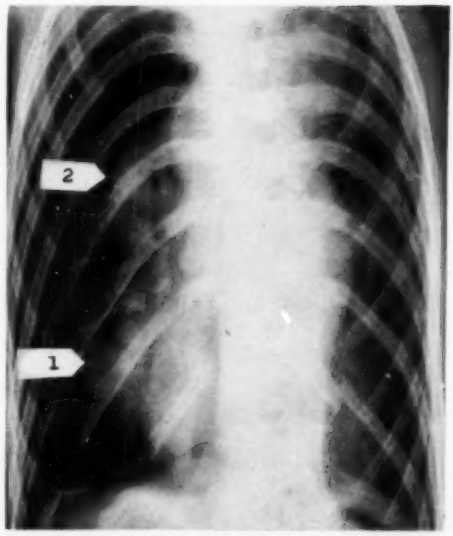

Fig. 1—Advanced pulmonary tuberculosis in a monkey (58) with radiopaque abscesses in the inferior lobe (1) and superior lobe (2) of the right lung.

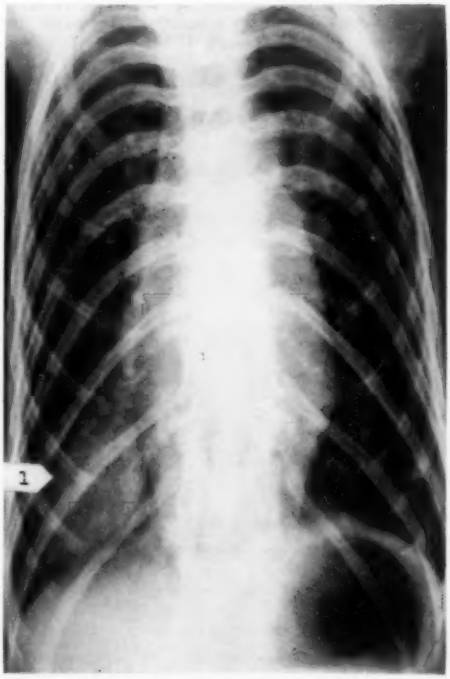

Fig. 2—Pulmonary tuberculosis in a monkey (77) showing a radiopaque inferior lobe of the right lung (1).

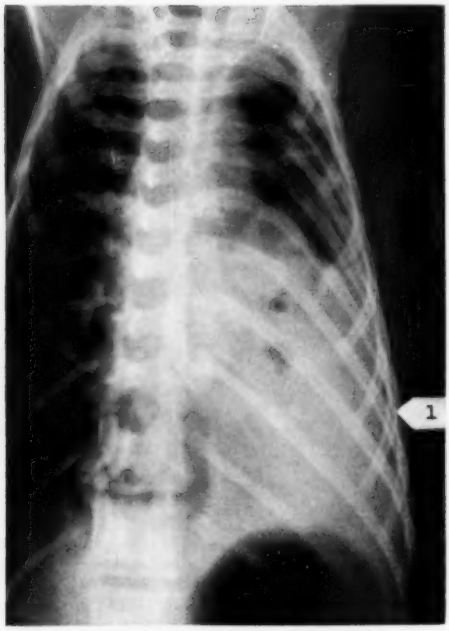

Fig. 3—Advanced pulmonary tuberculosis in a monkey (78) revealing a massive abscess in the left lung (1).

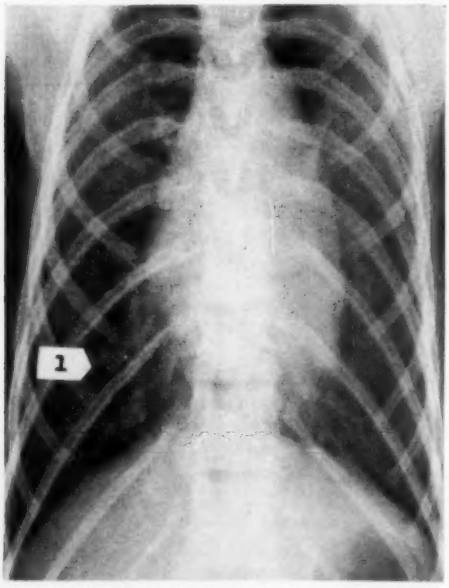

Fig. 4—Slight opacity in the inferior lobe of the right lung (1) of a nontuberculous monkey (82).

tagion of the other animals. A larger dose of tuberculin, as is now being used, might have resulted in a positive reaction in the tuberculous animals. Different investigators advocate dosages of tuberculin varying between 1 and 10 mg. of Old Tuberculin per injection.

One animal in colony I (82) was considered suspicious because two chest radiographs taken during the same week showed a slight opacity of the lower lobe of the right lung (fig. 4), although the tuberculin test was negative. However, no gross evidence of tuberculosis or pneumonia was found at necropsy. With further experience, it may become possible to distinguish more reliably between normal variations in opacity and tuberculous opacities.

It is not known whether the animals in colony II were free of tuberculosis at the time the first radiographs were taken and tuberculin tests made, or whether the tuberculin doses were too small to elicit a positive reaction. It is also possible that abdominal skin is less sensitive to the tuberculin antigen than is the eyelid.

The near lack of clinical signs of the infection in the 6 monkeys in colony II is of interest. In retrospect, project members recalled slight, infrequent coughing by 2 monkeys. The same 2 animals suffered some impairment in appetite two to three weeks before death.

In several animals, which had given no indication of ill health, massive tuberculous lesions were found in the lungs at necropsy. The signs of illness that were observed in 2 monkeys were not noticeably different from those occasionally seen, during the previous four years, in nontuberculous animals afflicted with upper respiratory infections or diarrhea, or which fasted voluntarily.

The occurrence of mesenteric lesions, in the absence of pulmonic lesions, in 3 animals in colony II, strongly indicates that the gastrointestinal tract was the route of infection. All animals were housed in separate cages which were stacked adjacent to one another. They were open at the front and back but the sides were solid metal and did not permit direct contact. The cages, arranged in two rows which faced each other, were separated by a distance of 5 feet. Thus, the direct spread of the organism by coughing or sneezing would seem to have been difficult. That it prob-

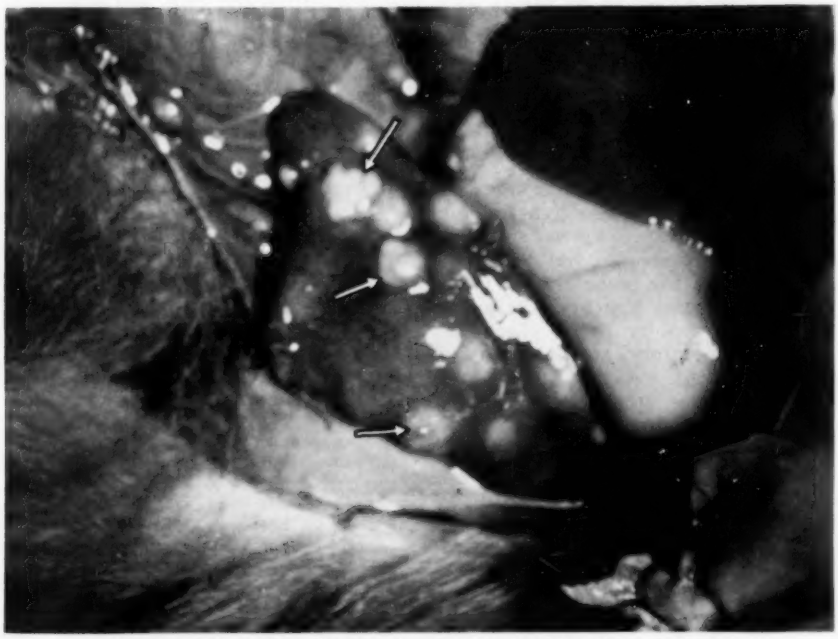

Fig. 5—Tuberculous abscesses (arrows) in the spleen of a monkey.

ably did occur is indicated by the presence of pulmonic lesions in 3 monkeys without abdominal lesions. However, the animals were fed and watered in small dishes which were taken out of the cages, refilled, and replaced in the cages daily. These containers were generally interchanged with no precaution taken to return each container to the cage from which it was taken. This was probably an important mode of transmission of the tubercle bacilli. The current animal care technique insures that each container is returned to its original cage and no interchange of food, water, or containers is permitted.

CONCLUSIONS

1) Lesions of pulmonary tuberculosis in monkeys can be easily demonstrated by chest radiographs when the disease is advanced.

2) Pulmonary tuberculosis in monkeys can sometimes be recognized by radiography early enough to permit removal from the colony before all the other animals become infected and sometimes before the disease has spread at all.

3) Radiography is a valuable aid to the palpebral tuberculin test, but not a substitute for it, in detecting advanced cases of pulmonary tuberculosis in experimental *Macaca mulatta* monkeys.

4) The interchanging of food and water containers may be an adequate means for the spreading of tubercle bacilli. It is, therefore, suggested that, for better control of tuberculosis in laboratory monkey colonies, the animal care techniques should be designed to insure against the interchange of food, water, or containers.

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The Effects of Heat and Hemoglobin on the Serum Proteins of the Guinea Pig

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IN THE PREPARATION of vesicular stomatitis and foot-and-mouth disease hyperimmune serums for use in the complement-fixation test, Brooksby [2] destroyed native complement by heating the serums at 56 C. for 30 minutes. Serums treated in this manner were analyzed by paper electrophoresis in this laboratory, and it was found that a component with the mobility of *alpha*-globulin was present in abnormal quantities. Hemolyzed samples showed a further increase in the quantity of this component. Because of these observations, a quantitative study was made to determine the effects of heating, addition of hemoglobin, and a combination of both factors on the components of normal guinea pig serum.

MATERIALS AND METHODS

Guinea Pig Serum.—Normal guinea pigs, weighing approximately 700 Gm. each, were deprived of feed for 24 hours and then exsanguinated. The blood was allowed to clot at 25 C. for two hours and the clots were then separated from the serum, which was stored at 4 C. and dispensed in vials in 20-ml. volumes. The vials were sealed and frozen at —40 C. until used. Serum from 200 guinea pigs was prepared in this manner. Any visibly hemolyzed samples were discarded.

Guinea Pig Hemoglobin.—Blood was collected from guinea pigs by cardiac puncture and diluted with modified Alsever's solution in equal volumes. The erythrocytes were centrifuged and washed four times with 0.85 per cent sodium chloride solution. The packed cells were then lysed by adding an equal volume of distilled water. Toluene (40 ml.) was added to 60 ml. of the lysate, and the mixture was shaken for 15 minutes to remove the lipids. The material was then centrifuged at 4,000 r.p.m. for 30 minutes, and the top two layers were removed by aspiration. The resulting hemoglobin solution was filtered through cotton and stored at —40 C. Fresh hemoglobin solutions were prepared after two months.

The concentration of hemoglobin was measured by spectrophotometric determination of total iron, as described by Fister.[3]

Serum-Hemoglobin Mixtures.—Hemoglobin concentrations of 1.7, 0.85, 0.43, 0.21, 0.1, and 0.05 per cent were prepared by making a 1:10 dilution of a tenfold concentration of hemoglobin with guinea pig serum. Portions of each mixture were heated in a water bath at 56 C. for 30 minutes.

Electrophoresis and Densitometry.—A horizontal type of paper electrophoresis apparatus, with a regulated constant current source, was used. Larson [5] found barbital buffer, at pH 8.6, satisfactory for hemoglobin analyses. Moore [7] used the same buffer for guinea pig serum. The buffer was prepared at 0.05 ionic strength.

The methods used were essentially those described by Block *et al.*[1] Schleicher and Schuell 598 filter-paper sheets, measuring 20 by 52 cm., were moistened with buffer and the two ends placed in the electrode vessels. Buffer and current equilibration was accomplished in approximately three hours. At this time, seven spots of 0.005 ml. each of serum, hemoglobin, or serum-hemoglobin mixtures were spaced, approximately 2.5 cm. apart, across the sheets and the current was allowed to flow at 1.5 milliamperes (ma.) for 21 hours at 25 C. The filter paper was dried at 100 C. for 30 minutes and stained for five minutes with a saturated alcoholic solution of mercuric chloride, containing 0.1 per cent bromphenol blue. The sheets were washed in distilled water for approximately two hours to remove uncombined dye and then dried at 25 C. Strips were cut and analyzed with a photoelectric densitometer equipped with a filter at 570 mμ. The percentages of serum components were calculated with a planimeter from plotted curves. Analyses for each sample were computed from seven strips.

RESULTS

The results of a quantitative study of the effects of heat and the addition of hemoglobin on the relative percentage composition of guinea pig serum, as analyzed by paper electrophoresis, are shown (table 1). Lesser concentrations of albumin and of the *beta*- and *gamma*-globulins and greater concentration of *alpha*-globulin were observed in heated serum than in unheated serum. When the concentration of hemoglobin exceeded 0.43 per cent in mixtures containing varying quantities of hemoglobin in unheated serum, a decrease

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in albumin and an increase in the *alpha*- and *beta*-globulins were observed, whereas the concentration of *gamma*-globulin remained unchanged. The results obtained with heated mixtures of serum and hemoglobin were quantitatively dissimilar. The effects of heating were more evident in

TABLE 1—The Effects of Heat and the Addition of Guinea Pig Hemoglobin on the Relative Percentages of the Components of Guinea Pig Serum

Hemoglobin (%)	Heated or unheated	Relative percentages of serum components			
		Albumin	Globulins		
			Alpha-	*Beta-*	*Gamma-*
0.00	Unheated	48.8	27.8	10.6	12.8
0.00	Heated	45.5	34.5	8.9	11.1
0.05	Unheated	49.2	27.2	10.9	12.7
0.05	Heated	46.0	34.3	8.9	10.8
0.10	Unheated	49.0	27.5	10.3	13.2
0.10	Heated	45.6	35.3	8.0	11.1
0.21	Unheated	48.4	27.6	11.2	12.8
0.21	Heated	44.3	35.8	9.4	10.5
0.43	Unheated	48.7	27.0	11.4	12.9
0.43	Heated	42.2	36.6	10.6	10.6
0.85	Unheated	47.4	28.5	11.7	12.4
0.85	Heated	39.3	38.9	10.9	10.9
1.70	Unheated	42.3	31.3	13.8	12.6
1.70	Heated	34.8	42.3	12.1	10.8

mixtures containing more than 0.21 per cent hemoglobin when compared with the same mixtures when unheated. There were significant decreases in albumin and increases in *alpha*-globulin, while *beta*-globulin increased slightly. The concentration of *gamma*-globulin was not influenced significantly by the various concentrations of hemoglobin. The slight decrease appeared to be attributable to heating.

Electrophoretic mobilities of hemoglobin solutions tested before and after heating by paper electrophoresis were identical. It was also observed that hemoglobin was a homogeneous substance which was not affected by heating under the conditions described. There were no significant differences in mobility of the individual serum components between unheated or heated serum-hemoglobin mixtures.

DISCUSSION

Paper electrophoretic analyses of guinea pig hemoglobin in barbital buffer solution at pH 8.6, 0.05 ionic strength, showed one component with the mobility of *alpha*-globulin. When hemoglobin was heated at 56 C. for 30 minutes, it was still seen as a homogeneous substance, the only apparent difference being an insignificant increase in optical density, which was probably due to molecular aggregation and an increased affinity for the bromphenol blue dye.

On the other hand, when guinea pig hemoglobin was added to guinea pig serum, increases in *alpha*- and *beta*-globulins occurred, as analyzed by paper electrophoresis in the fresh state. Increases in the concentrations of these two serum components indicated that hemoglobin, which has the mobility of a single entity, formed complexes with *alpha*- and *beta*-globulins. This has been suggested, but conflicting data have been presented, possibly due to the different test methods and conditions used. The results have indicated either that the effect of hemoglobin in human and bovine serums is additive to a serum component, because it may possess a similar mobility, or that a complex or combination occurs.[4, 8-13] Studies of human serum with added hemoglobin, as reported by Moore *et al.*,[8] revealed an increase in the *gamma*-globulin concentration when such serum was analyzed in phosphate buffer at pH 7.4. In phosphate-chloride buffer, at pH 7.7, the $alpha_2$-globulin increases when hemoglobin is added to serum, as reported by van Royen *et al.*[13] In similar experiments, performed in barbital buffer at pH 8.6, the *alpha*-globulins[4, 9, 11, 12] and the *beta*-globulins[8, 9, 11, 12] increased when hemoglobin was added.

In tests reported here, guinea pig serum, heated in a water bath at 56 C. for 30 minutes and subsequently analyzed electrophoretically, in barbital buffer at pH 8.6, 0.05 ionic strength, showed decreases in concentrations of albumin and *beta*- and *gamma*-globulins and an increase in *alpha*-globulin. Moore *et al.*[8] have shown that, when human serum was heated at 58 C. for 20 minutes and then analyzed in phosphate buffer at pH 7.4, an increase occurred in the *alpha*-globulin, with a decrease in albumin and *beta*-globulin. According to Leyton,[6] variable results were obtained when guinea pig serum was heated at 56 C. for 30 minutes, although the *alpha*-globulin concentration generally increased when tested in phosphate-sodium chloride buffer at pH 7.4. As reported by van Royen *et al.*,[13] human serum, in phosphate-sodium chloride buffer at pH 7.7, when heated at 55 C. for 20 minutes,

showed increases in the *alpha-* and *beta*-globulins.

Although there was a slight increase in dye-binding capacity of heated hemoglobin in the writers' tests, this apparently was not sufficient to account for the increase in *alpha*-globulin concentration, when measured with a photoelectric densitometer. It appears that heating, under the conditions described, increases the formation of a complex in serum-hemoglobin mixtures. It has been suggested that the substance forming a complex with hemoglobin is haptoglobin, which is present in the $alpha_2$-globulin.[4, 11, 13] Haptoglobin has been reported to lose its catalytic power, when heated at 60 C., after a short period of time.[13] From the data presented here, it is apparent that significant errors may occur when guinea pig serum is analyzed by paper electrophoresis under the conditions described. Increases were observed in the *alpha*-globulin, with decreases in the albumin, when guinea pig serum was heated, if it contained hemoglobin, or if the guinea pig serum-hemoglobin mixtures were heated.

SUMMARY

1) Guinea pig serum, hemoglobin, and serum-hemoglobin mixtures, before or after heating at 56 C. for 30 minutes, were subjected to electrophoresis on filter paper in barbital buffer, at pH 8.6, at 0.05 ionic strength.

2) Alterations occurred in the serum protein composition when the serums were heated at 56 C. for 30 minutes. There were decreases in the concentrations of albumin and *beta-* and *gamma*-globulins, while the concentration of *alpha*-globulin increased.

3) A decrease in albumin was evident when guinea pig hemoglobin was added to fresh serum. Increases in the concentrations of *alpha-* and *beta*-globulins occurred, while the *gamma*-globulin concentration was not affected.

4) When hemoglobin was analyzed electrophoretically, it appeared as a homogeneous component having the mobility of *alpha*-globulin. The increases in concentration of both *alpha-* and *beta*-globulins of the serum-hemoglobin mixtures did not coincide with the homogeneous electrophoretic mobility of hemoglobin alone. Therefore, it appeared that complexes were formed, mainly between *alpha*-globulin and hemoglobin and, to a lesser degree, between *beta*-globulin and hemoglobin.

5) Heating of the serum-hemoglobin mixtures seemed to result in an increase of the formation of a complex.

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Primary Isolation and Comparative Titrations of Five Field Strains of Vesicular Stomatitis Virus in Chicken Embryos, Hogs, and Mice

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VESICULAR STOMATITIS occurs as an enzootic disease of swine, cattle, horses, deer, and raccoons on the coastal plain of the southeastern United States.[4] A confirmed diagnosis of vesicular stomatitis in animals other than horses and cattle has never been made in epizootics of the disease in other parts of North America.[2] The prevalence of the disease in swine and wildlife appears to be a phenomenon peculiar to the enzootic area. It was this observation which led to an interest in the host predilections of strains of virus involved in the annual outbreaks on the coastal plain.

Five samples of vesicular epithelium were received in December, 1955, from the Virus Research Laboratory of the Agricultural Research Service at Beltsville, Md. These tissues had been harvested from animals involved in enzootics of vesicular stomatitis in swine and cattle which had occurred the previous summer in the states of North Carolina, South Carolina, Georgia, and Louisiana. In one case (Sweatman strain), the tissue sample was from a horse which had been used in establishing the diagnosis. All tissues had been stored in a dry ice chest in sealed vials with buffered glycerol. The virus contained in each sample had been identified as vesicular stomatitis virus, New Jersey serotype (NJ), by complement-fixation tests conducted at Beltsville. A list of the viral strains and the source of each is shown (table 1). A sixth strain (Hazlehurst), which had been propagated in the chicken embryo after its initial isolation from swine in 1952, was included in comparative titrations. It had been stored as lyophilized allantoic fluid from infected chicken embryos. In addition to these southern isolates, the Wisconsin strain of NJ-type vesicular stomatitis virus, which had been propagated in chicken embryos after its isolation from a cow in 1949, was used for comparative titrations in chicken embryos and cattle.

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METHOD

The five virus-bearing samples of epithelium were ground with ten times their volume of sterile tryptone broth in TenBroeck grinders. Resultant suspensions were centrifuged at 3,000 r.p.m. for ten minutes. Crystalline potassium penicillin G and crystalline dihydrostreptomycin were added to the supernatant fluids to make final concentrations of 2,500 units and 2,500 μg./ml., respectively. Further serial tenfold dilutions to 10^{-4} were made from each virus stock using tryptone broth. Similar dilutions were prepared by reconstitution of the lyophilized sample of the Hazlehurst strain.

Six white crossbred pigs, each weighing about 40 lb., were used for the titration in swine. Preinoculation blood samples were taken for serum-neutralization tests. One virus isolate was used per pig, 0.1 ml. of one of the tenfold dilutions being inoculated intradermally into the coronary band of each foot, so that all four dilutions were used on each pig. In each case, the 10^{-1} dilution was also inoculated intradermally on the snout. Preinoculation blood samples were drawn from the anterior venae cavae. Rectal temperatures were recorded daily for each animal. All signs of disease were recorded.

The same viral dilutions, with the exception of 10^{-4}, were inoculated intracerebrally into young adult white mice (Carworth farms stock of Rockefeller Swiss Albino mice—CFW), using 0.03 ml. per inoculum and 6 mice per dilution. Simultaneously, all four serial dilutions of each virus isolate were inoculated by the allantoic route into 8-day embryonated eggs, using 0.1 ml. per inoculum and 6 eggs per dilution.

RESULTS

Preinoculation serum samples from the swine were found negative for antibodies against NJ-type vesicular stomatitis virus. Body temperatures were found to be slightly elevated 24 hours postinoculation and decidedly elevated at 48 hours postinoculation. In most of the animals, a somewhat diphasic temperature response occurred with peaks on the second or third

TABLE 1—Strains of New Jersey-Type Vesicular Stomatitis Virus Used in Comparative Titrations

Strain	Animals involved in enzootic	Date of collection	Locality
Adamson	Swine	7/29/55	Tattnall Co., Ga.
Avoyelles	Swine	5/24/55	Avoyelles Parish, La.
Bailey	Swine	8/26/55	Wayne Co., Ga.
Hazlehurst	Swine *	5/16/52	Jeff Davis Co., Ga.
Sweatman	Swine †	6/ 5/55	Orangeburg Co., S. Car.
Wooten	Cattle	8/ 9/55	Columbia Co., N. Car.
Wisconsin	Cattle ‡	8/..../49	Rusk Co., Wis.

* Originally isolated from swine, the source of the inoculum was allantoic fluid from the third chicken embryo passage.

† Originally isolated from swine, the source of the inoculum was vesicular epithelium from a horse used in establishing the diagnosis.

‡ Originally isolated from cattle, the source of the inoculum was allantoic fluid from about the twelfth chicken embryo passage.

days and again on the fifth to seventh days after inoculation (table 2).

Throughout the course of the infection, the animals remained alert and ate fairly well, although lameness, which was pronounced in 2 of the animals (S3 and S6), restricted activity somewhat. Lesions, in the form of swelling and blanching of the coronary bands, first became apparent about 48 hours after inoculation. By 72 hours postinoculation, vesicles were evident and at this time the titrations were read. Lesions seemed to be of an "all-or-none" type, inasmuch as they encircled the entire coronet on those feet which became affected. Lesions between the claws were either slight or absent. There appeared to be no formation of secondary lesions or tendency toward generalization. Snout vesicles were larger, contained more fluid, and were much more delicate and easily ruptured than were the more deeply situated foot vesicles. Vesicles on the coronet were long and narrow, being about 1 cm. in width and encircling the foot.

Depression, progressive paralysis, and death were observed in the infected mice. The deaths occurring from the third to the sixth day after inoculation were considered specific in the absence of demonstrable bacterial contaminants by culture on thioglycollate broth and nutrient agar. Titrations were calculated in 50 per cent lethal doses ($l.d._{50}$) of virus.

Infected chicken embryos died chiefly between 24 and 60 hours after inoculation. Allantoic fluids were harvested and found negative for bacterial contamination when cultured in thioglycollate broth and on nutrient agar. Chicken embryo titrations were also expressed in $l.d._{50}$ units of virus.

Convalescent serum samples were taken from all 6 pigs on the twenty-fifth day after exposure. At this time, all serums neutralized 10,000 or more $l.d._{50}$ units of NJ-type vesicular stomatitis virus in 10-day embryonating chicken eggs.[1] All pigs made uneventful recoveries.

The results of pig, mouse, and chicken embryo titrations are summarized (table 3).

DISCUSSION AND CONCLUSIONS

It has been postulated that host adaptation of vesicular stomatitis virus is readily induced.[6] For this reason, an effort was made to obtain stocks of virus in epithelial tissues harvested from animals involved in field infections. In this way, initial isolation could be made simultaneously in a number of different hosts and the titers so obtained in these hosts could be used to characterize the strains. The two chicken embryo propagated strains were included in comparative titrations to determine, if possible, changes in strain characteristics which might be brought about by such methods of cultivation of the virus.

Evidence was obtained of minor differences among strains but there was no clear distinction between cattle and swine strains. The Wooten strain from cattle, when inoculated intradermally, had as

TABLE 2—Swine Temperature Records (F.)

Animal No.	Strain inoculated	Days postinoculation										
		1	2	3	4	5	6	7	8	9	10	11
S1	Wooten	103.8	105.6*	105.8	104.4	105.4	104.0	104.0	103.1	102.8	103.1	102.6
S2	Sweatman	103.1	104.9	104.9	104.4	104.0	104.9	103.1	102.8	103.0	103.1	103.0
S3	Bailey	103.6	106.3	104.5	104.7	105.3	104.0	104.9	104.2	104.4	103.6	103.6
S4	Avoyelles	103.8	104.9	104.2	103.1	103.5	103.1	102.6	102.6	103.2	102.8	103.4
S5	Adamson	104.5	104.2	104.2	103.5	103.6	104.4	104.5	102.6	103.6	104.0	103.6
S6	Hazlehurst	103.3	106.6	104.9	104.2	106.3	106.0	104.2	103.6	103.6	104.5	102.6

* Recognizable peak temperatures are underlined.

TABLE 3—Results of Virus Titrations* in Swine, Mice, and Chicken Embryos

Strain	Initial source	Source of inoculum	Titer in swine	Titer in mice	Titer in chicken embryo
Adamson	Swine	Initial sample	$10^{4.0}$	$3 \times 10^{4.0}$	$10^{5.0}$
Avoyelles	Swine	Initial sample	$10^{4.5}$	$3 \times 10^{4.5}$†	$10^{2.6}$
Bailey	Swine	Initial sample	$10^{5.0}$†	$3 \times 10^{4.5}$†	$10^{5.5}$†
Hazlehurst	Swine	Chicken embryo (3rd passage)	$10^{4.0}$	$3 \times 10^{4.5}$†	$10^{5.5}$†
Sweatman	Swine	Horse (1st passage)	$10^{4.0}$	$3 \times 10^{4.5}$†	$10^{5.5}$†
Wooten	Cattle	Initial sample	$10^{5.0}$†	$3 \times 10^{4.5}$†	$10^{2.5}$
Wisconsin ‡	Cattle	Chicken embryo (10th-20th passage)	$10^{5.0}$	10^{5}–10^{6}	10^{6}–10^{7}

* Swine titers are presented as the number of infective doses per milliliter of inoculum. Mouse and chicken embryo titers are presented as the number of 50 per cent lethal doses per milliliter of inoculum.
† Titers in which the end point was not reached because higher dilutions were not tested.
‡ Results obtained previously included here for comparative purposes.

great an infectivity for swine as any of the swine strains. The behavior of the Hazlehurst strain, isolated from swine but subsequently adapted to the chicken embryo, was not found to differ markedly from that of the new isolations on initial passage in hogs, mice, and chicken embryos.

Titration end points, unfortunately, were not reached in some instances because higher dilutions were not tested. This was due to an error in estimation of the probable infectivity of the stored tissues. It was indeed surprising to find such high titers of virus, in new hosts, with vesicular epithelium which had been stored for periods up to seven months. Such results are a tribute to the field personnel who collected and preserved the materials.

It is unfortunate that titrations in the bovine tongue could not have been attempted simultaneously with the hog, mouse, and chicken embryo titrations. At a later date, second chicken embryo passage allantoic fluids of the Adamson and Avoyelles strains titrating 10^5 $l.d._{50}$ units of virus per milliliter in chicken embryos, and the chicken embryo-propagated Wisconsin strain titrating $10^{5.5}$ $l.d._{50}$ units per milliliter, were used to expose 2 heifers, 2 years old, by intramucosal inoculation of the tongue. The Wisconsin strain was used on both animals, being compared with the Adamson and Avoyelles strains inoculated in tenfold serial dilutions on opposite sides of the tongue. Titrations were read at 48 hours as 10^3 infective doses of virus per milliliter for each of the three strains on the basis of the appearance of vesicles at the sites of inoculation. Similar titrations of the Adamson strain in 2 deer yielded titers of 10^6 infective doses of virus per milliliter.[5] This strain, on initial isolation from vesicular epithelium, reached a titer of 10^4 infective doses per milliliter in swine.

It seems, on the basis of these observations, that this strain (Adamson) may have been adapted to growth in deer in preference to swine and cattle. However, due to the observed behavior of a strain of bovine origin (Wooten) which grew well in swine, it seems unsafe to draw conclusions as to the natural host predilection of virus strains until more complete studies have been made. In support of the theory of "host adaptation" through passage, the chicken embryo-propagated Hazlehurst strain reached higher titers in chicken embryos than it did in swine or mice, and the similarly propagated Wisconsin strain reached a higher titer in chicken embryos than it did in cattle.

Contrary to a previous supposition,[3] the chicken embryo was found to be a suitable host for primary isolation of vesicular stomatitis virus. Only two of the strains used in this experiment yielded chicken embryo titers below the titers obtained in swine, in spite of the fact that four of the five strains of which primary isolations were made were from swine. These observations, plus the low cost of eggs, the ease of handling, and economy of space, should recommend chicken embryos for use in diagnostic procedures requiring indicator hosts with uniformly high susceptibility to field strains of vesicular stomatitis virus.

SUMMARY

The occurrence of vesicular stomatitis in swine, deer, and raccoons in the southeastern states, in the absence of recognized disease in these animals in epizootics in other parts of the country,[2] suggests differences in the host predilection of the strains of virus involved in enzootics and epizootics. To examine this possibility, five strains of virus obtained as vesicular epithelium from animals involved in recent

infections were compared, by simultaneous titration in swine, mice, and chicken embryos, with each other and with a chicken embryo-propagated strain. Two of these field strains and the chicken embryo-propagated strain were later titrated in cattle, and one of the strains was also titrated in deer. Minor differences in the affinity of the strains for certain hosts were found but these could not be related to initial isolation from a particular host. The Adamson strain isolated from swine showed a one thousandfold higher titer in deer (10^6 i.d.) than in cattle (10^3 i.d.), and a tenfold higher titer in chicken embryos (10^5) than in swine (10^4). Replicate titrations, not possible with the small initial sample available, would be necessary before the differences could be considered significant.

Host adaptation may occur to a limited extent, as the chicken embryo-propagated strains reached higher titers in their accustomed host than they did in swine or mice, although these differences were small.

The chicken embryo was found to be a sensitive host for initial isolation and titration of field strains of vesicular stomatitis virus. It may be recommended for use in diagnostic procedures.

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Effects of Hypotonic and Hypertonic Sodium Chloride Solutions on First-Instar *Hypoderma Lineatum* Larvae

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THIS REPORT presents information on the semipermeability of the body wall of first-instar *Hypoderma lineatum* larvae and the *in vitro* effects of hypotonic and hypertonic sodium chloride solutions on this parasite. It also contains a suggestion as to the possible use of sodium chloride as a means of raising the osmotic pressure of the extracellular body fluids of the host and, thereby, destroying the parasite.

MATERIALS AND METHODS

The tests reported were conducted from June through December, 1956, in the Grand Rapids area, with larvae isolated from commercially slaughtered Hereford heifers and steers, 8 to 24 months of age. Within two to three hours after the cattle were killed, larvae were removed from the esophagi and placed in Ringer's solution at a temperature of approximately 65 to 78 F. Only larvae of one esophagus were used for one series of tests. The testing usually started four to five hours after the cattle were killed. The larvae were transferred to a Petri dish, half filled with Ringer's solution, and examined under 40-power magnification. The immobile or slowly moving larvae were discarded. Apparently vigorous larvae were transferred to covered Petri dishes half filled with the various solutions, the control larvae being left in Ringer's solution. Checks of larvae were made at certain intervals. After the larvae in any of the solutions ceased to exhibit movements of any kind, they were transfered to Ringer's solution, since motionless, apparently dead larvae sometimes recovered following such transfers. Attempts were also made to activate some larvae by slight warming of the solutions.

RESULTS AND DISCUSSION

The data on some typical tests are summarized (table 1). The survival times obtained from many tests varied, although they were conducted with larvae of approximately the same size and under similar conditions. This was probably due to the various degrees of resistance possessed by individual larvae or their groups. From tests involving hundreds of larvae, it could be concluded that, in the hypertonic solutions, the time required to kill the larvae was directly proportional to their size and in inverse proportion to the salt concentration and temperature of the solution. These facts suggest that the younger the larvae, the more they might be susceptible to changes in the osmotic pressure of the body fluids of the host.

It is apparent from these tests that the body wall of the larva acts as a semipermeable membrane which readily allows water molecules to pass through it in either direction, but prevents the passage of particles of larger size, e.g., sodium and chlorine ions. The body wall of the larva probably allows nothing but water to go through it. It seems that, normally, the osmotic pressures between the body fluids of the larvae and those of the host are at equilibrium. In the hypotonic and hypertonic solutions, the destruction of larvae results from undue amounts of water being drawn either into or out of their bodies by osmosis. Although the only agents tested were common salt and dextrose, there seems to be little doubt that changes in the osmotic pressure of the solutions, induced by some other means, would affect larvae similarly.

Further studies will be required to appraise the value and significance of this apparent vulnerability of *H. lineatum* larvae. The fact that the larvae possess this attribute suggests the possibility of increasing the osmotic pressure of the body fluids of the host animal by administration of a maximal dosage [11] of sodium chloride to a degree and for a period time sufficient to destroy the parasite without causing any appreciable damage to the host.

Cattle that are not restricted in their water intake may ingest a relatively high dosage of common salt without showing signs of intoxication and the marked increase in concentration of chlorides in their blood remains for many hours.[10] The osmotic pressure of the body fluids is increased by the high chloride levels of the blood.[3,7] Since Hypoderma larvae are actually immersed in the extracellular fluids of the host, loss of this fluid due to in-

The author is a veterinary meat inspector. These observations were made without the advantages of an adequate library or laboratory and with no financial aid.

TABLE 1—Effects of Sodium Chloride Solutions on Survival Time of Hypoderma Lineatum Larvae

Solution	Approx. temp. of sol. (F.)	No. larvae measured	Size of larvae		Survival time			
			Mean length	Range of length	In test sol.* (hr.)	(min.)	In Ringer's sol.† (hr.)	(min.)
Tap water	69	18	10.5 mm.	8–13 mm.	0	16	25	0
Tap water	100	8	4.5 mm.	4– 5 mm.	0	2	3	0
Tap water	100	22	10.5 mm.	8–13 mm.	0	4	6	0
0.55% NaCl	69	12	6.5 mm.	5– 8 mm.	14	0	20	0
1.10% NaCl	62	12	12.5 mm.	10–15 mm.	30	0	96	0
1.10% NaCl	67	11	10.5 mm.	7–14 mm.	17	0	27	0
1.10% NaCl	102	4	4.5 mm.	4– 5 mm.	3	40	4	30
1.10% NaCl	104	12	9.5 mm.	8–11 mm.	3	0	5	0
1.25% NaCl	69	12	6.5 mm.	5– 8 mm.	10	0	20	0
1.25% NaCl	70	16	8.5 mm.	7–10 mm.	4	30	20	0
1.50% NaCl	69	12	6.5 mm.	5– 8 mm.	10	0	20	0
1.50% NaCl	75	15	11.0 mm.	9–13 mm.	5	0	7	0
1.80% NaCl	70	20	8.5 mm.	7–10 mm.	1	30	20	0
1.80% NaCl	102	6	4.5 mm.	4– 5 mm.	0	30	3	0
Dextrose 10% in saline sol.	75	12	8.5 mm.	5–12 mm.	0	15	5	0

* All larvae showed no signs of life after the time indicated. In tap water, their body walls ruptured following immense swelling. In strong hypertonic solutions (1.80%) death was preceded by marked shrinkage of body.

† Some signs of life were still visible in 1 or more controls after the time indicated. Temperature was alike in both test and Ringer's solutions.

creased concentration of sodium chloride in the blood [9] would probably draw water from the bodies of the larvae and affect them unfavorably.

Trials to determine the systemic effectiveness of sodium chloride against cattle grubs *in vivo* would be relatively simple and inexpensive since much is already known about dosage, toxicity, effect on blood levels, and rate of elimination. Tests using 1 animal would suffice to determine the magnitude of the increase in osmotic pressure of body fluids after ingestion of a maximal dosage of sodium chloride. The effectiveness of serum with a high content of chlorides could be tested on the larvae isolated from the esophagi of freshly killed cattle. Methods for such testing seem to be well elaborated.[1]

If the increased osmotic pressure remained at a comparatively high level for several hours, and if the larvae appeared affected by serum containing a high concentration of chlorides, a firm basis would be established for trials with cattle presumably infected with young Hypoderma larvae.

Since *H. lineatum* larvae accumulate in the esophagus and periesophageal tissues for several weeks during one period of the year, it might be of interest to determine the feasibility and effectiveness of infiltrating the cervical region with strong sodium chloride solutions in an attempt to destroy the larvae. The determination of the effects, if any, of salt as a feed inhibitor on the development of the first-instar Hypoderma larvae might be of great practical value. The osmotic pressure of the body fluids of animals regularly ingesting relatively high amounts of salt is increased well above normal levels, and this might possibly affect the larvae. An attempt should also be made to determine the synergetic effectiveness of large doses of salt together with some other parasiticides, such as phenothiazine.

SUMMARY

The semipermeability of the body wall of first-instar *Hypoderma lineatum* larvae is reported.

In vitro tests with hypotonic and hypertonic sodium chloride solutions indicate that the time required to kill the larvae varies directly with their size and inversely with the concentration and temperature of the solution.

The possibilities for the peroral and parenteral use of sodium chloride for the systemic destruction of first-instar Hypoderma larvae *in vivo* are suggested.

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A Modified Erlenmeyer Flask for Use in Culturing Entire Yolks of Turkey Eggs

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THIS PAPER describes a method of obtaining entire turkey egg yolks, in a sterile manner, for culture purposes.

PREPARATION OF THE FLASK

An area on the side of a 300-cc. Pyrex Erlenmeyer flask, 2½ inches below the lip, is ground to, but not through, the inner surface. A rapidly turning emery wheel is satisfactory for this purpose. The hole produced is enlarged with a Burgess vibrograver until a rubber stopper of the type used in bottles for sterile diluent vaccine and similar preparations will fit tightly in the hole. Any of the stoppers identified as follows may be used: West S-46, Carron 312, U.S. Rubber 2771, and Faultless 5020.

A ¾-inch, 19-gauge hypodermic needle is inserted through the rubber stopper so that a syringe can be attached from the outside of the flask and air can be aspirated from the inside. A cotton stopper should be inserted into the mouth of the flask and the upper portion, including the inserted needle (fig. 1), should be wrapped in brown paper and the entire apparatus pressure sterilized.

OPERATION OF THE FLASK

A turkey egg is secured with sterile Fisher safety flask tongs and placed in a beaker of ethyl alcohol. The egg is again secured with the tongs and held over a Bunsen burner until the alcohol is ignited. As soon as it has burned dry, the egg is placed (large end up) on a sterile Coplin staining jar. The large end of the egg is

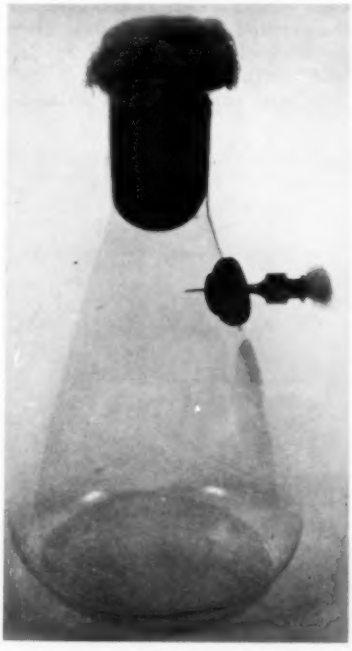

Fig. 1—Completed flask, ready for sterilization.

From the Clemson College Livestock Sanitary Department, Columbia, S. Car.

The perfection and use of the flask herein described is an outgrowth of an egg culturing technique supplied by Dr. J. E. Williams, chief of the Program Services Section, Agricultural Research Service, Animal Disease Eradication Branch, U.S. Department of Agriculture.

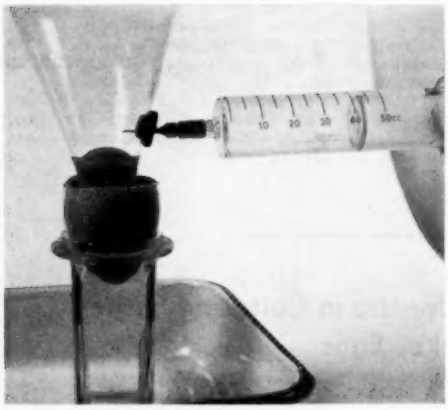

Fig. 2—Egg yolk being aspirated up into flask.

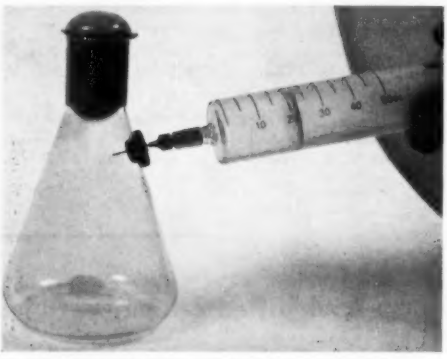

Fig. 3—The removed yolk in the flask.

burned with an improved Fisher burner until the shell is black. The burned pieces of shell may be removed with forceps or the burned portion may be cut around with sterile, sharp-pointed scissors until the inner contents of the egg are exposed.

A 50-cc. syringe is attached to the hypodermic needle of the modified flask. The flask is inverted and the lip is placed next to the egg yolk surface. Suction is applied with the syringe until the yolk is drawn up into the neck of the flask (fig. 2). Slowly, the flask, containing the yolk, is removed from the egg and returned to a normal position (fig. 3), allowing the yolk to drop to the bottom of the flask. Occasionally, a yolk will tend to slip back into the egg. When this happens, it is necessary to turn both the apparatus and the egg to a horizontal position and to obtain the yolk with a scooping action. With a little practice, one will learn to secure a yolk promptly.

Sterile broken glass is then placed in the modified flask and the yolk is triturated. Culture mediums can be poured into the flask or the triturated contents can be poured into another flask containing sterile culture mediums. Sterile technique is used at all times.

Bacteriophage Typing of Micrococci of Bovine Origin

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IN RECENT YEARS, bovine mastitis caused by *Micrococcus pyogenes* has become more common in Wisconsin. In an attempt to develop a method for controlling the spread of this infectious agent, an epizootiological study was undertaken. It was conducted, in part, by means of phage typing which has been shown to be of value in following the infectious agents through populations in epidemiological studies.[2, 6]

Phage typing is based on the determination of the sensitivity or resistance of a particular bacterial isolate to several phages. The data obtained constitutes the phage type of the isolated organism. For example, Blair and Carr [2] showed that a majority of the cultures that were isolated from environmental sources in the nursery of a hospital, and from the nasopharynx or skin of infants and attending nurses, were of the same phage type, but cultures from various infections in other wards of the same hospital were of other phage types. They concluded that an endemic focus of infection existed in the nursery. In a similar study, Jackson *et al.*[6] showed that the hospital personnel played a role in acquiring and disseminating *M. pyogenes* among the patients.

One of the early studies on the role of Micrococcus phages in bovine mastitis was done by Slanetz and Jawetz,[12] who isolated phages from milk and, with these phages, attempted to classify the bacterial cultures also isolated from milk. Subsequently, Williams-Smith [16] made the first extensive study on the applicability of phage typing for epizootiological studies and concluded that it may have limitations in epizootiological studies of bovine mastitis. Some of these limitations of the present phage typing method [15, 16] will be discussed.

MATERIALS AND METHODS

Culture Mediums.—Nutrient broth and agar (Albimi) and yeast extract peptone broth and agar were used. The latter medium consisted of 3 Gm. of yeast extract (Albimi), 5 Gm. of peptone (Bacto), and 5 Gm. of sodium chloride per 1,000 ml. of distilled water plus agar. Much of our early work was done with the use of the nutrient medium, but we now routinely use the yeast extract peptone medium because it gives more uniform growth of *M. pyogenes*. Yeast extract peptone agar slants and broth (pH 7.2 to 7.4, 1.5% agar) were used for culturing, and this same agar medium was used for base layers of agar. Soft agar medium (pH 8, 0.7 to 0.8 agar) was used for the routine typing. The pH of the mediums was always adjusted with sodium hydroxide prior to autoclaving.

Phages.—The 19 phages of the International Series (phages 29, 52, 52A, 79, 3A, 3B, 3C, 55, 6, 7, 42E, 47, 53, 54, 70, 73, 75, 77, and 42D) and six other phages (39, 523, 42B, VA4, 44A, and 81), and the propagating strains for these phages, were received from Blair.* Twelve phages (31, 44, 51, 47A, 47B, 47C, W, 977, 1272, 1791, 971, and 142) and the propagating strains for these phages were received from Jackson.† The phages listed listed above have been used primarily for typing micrococci of human origin.[2, 6, 15]

Six phages, S1, S2, S3, S4, S5, and S6, were isolated in our laboratory and were propagated on micrococci of bovine origin.[11] Seven phages were selected (table 1) for phage typing cultures of bovine origin, partly because not many of these cultures were lysed by some of the phages that have been used for typing micrococci of human origin.

TABLE 1—Seven Phages and Their Propagating Strains, Origins, and Serotypes

Phage	Propagating strain	Phages from which each was derived	Serotype
42D	1363	.....	B
S1	10	42D	B
S2	11	42D	B
S3	12	.....	B
S4	13	S3	B
S5	14	S3	B
S6	15	S3	A

At the start of our investigation, we did not have all of the 19 phages of the International Series. Five of the seven phages (table 1) were derived from phages 42D and S3. Phages S1 and S2 were derived from phage 42D, and phages S4, S5, and S6 were derived from phage S3.[11] The

From the Department of Bacteriology, University of Wisconsin, Madison. Published with the permission of the director of the Wisconsin Agricultural Experiment Station.

The authors are grateful to Mrs. Nancy J. Fink for technical assistance.

* Dr. John E. Blair, Hospital for Joint Diseases, New York, N. Y.

† Dr. G. G. Jackson, University of Illinois, Chicago.

TABLE 2—The Host Range of the Seven Phages, as Tested on the Seven Propagating Strains at the Routine Test Dose (RTD)

Culture	Phage						
	42D	S1	S2	S3	S4	S5	S6
1363	CL*	—	—	—	—	—	—
10	—	CL	—	CL	—	—	—
11	—	—	CL	—	—	—	—
12	—	—	—	CL	—	—	—
13	—	—	—	—	CL	—	CL
14	—	—	—	—	—	CL	—
15	—	—	—	—	—	—	CL

* CL = Confluent lysis; — = no reaction.

serotypes of the phages listed (table 1) were determined by the method of Rountree.[10] Phages S1, S2, S3, S4, S5, and 42D were determined as serotype B, and phage S6 was serotype A. Rountree[9] reported that, as a rule, serotype B phages were active against micrococci of bovine and human origin, and that serotype A phages were active only against micrococci of human origin. The host range of the seven phages, as tested on the seven propagating strains, is given (table 2). Five of the seven phages lysed only their propagating strains at the routine test dose (RTD). Two phages, S3 and S6, lysed both their propagating strain and one other strain.

Cultures.—From Simon,** we obtained 380 coagulase-positive, *beta*-hemolytic and 50 coagulase-negative Micrococcus cultures. The cultures were isolated from quarter milk samples which were taken from cows in the Madison, Wis., area. The coagulase test was done with rabbit plasma, and hemolysis was observed on bovine blood agar plates.

From Thorsell‡ we received 34 coagulase-positive cultures of *M. pyogenes*, which were isolated from milk in Sweden.

Phage Typing.—Initially, the phages were propagated by the agar layer method of Swanstrom and Adams,[12] as adapted to the Micrococcus phage system by Jackson *et al.*[6] Once phage suspensions of relatively high titer (10^8 to 10^9 particles per ml.) were obtained, they were routinely propagated by the broth method.[11] Phage titers were determined by standard phage methods[1] and, for phage typing, the routine test dose (RTD) was determined by the method of Williams and Rippon.[15] The RTD is the greatest tenfold dilution of the phage suspension which gives confluent lysis of the indicator strain.

A combination of the three related techniques for phage typing, as described by Williams and Rippon,[15] Blair and Carr,[2] and Jackson *et al.*,[6] was adopted. Soft agar medium (0.7 to 0.8% agar) plates were poured a day ahead of time and left at room temperature overnight. Then the surface of the agar medium was swabbed, with the aid of a cotton applicator stick, with an 18- to 24-hour broth culture of the organism to be typed. The plates were then placed in a 37 C. incubator for about 60 minutes, to permit drying of the inoculated surfaces. This was accomplished by placing the plates bottom side up and ajar. After the plates were dry, the bottom sides were marked with a grid (fig. 1), by a special rubber stamp. The phages were spotted onto the cultures with 1.0-ml. tuberculin syringes equipped with a 27-gauge hypodermic needle. After the spots were dry, the plates were incubated at 30 C. for 14 to 18 hours. Results of phage typing were recorded as described by Jackson *et al.*[6] Figure 1 shows a typical plate after 14 to 18 hours of incubation. Notice the areas of confluent lysis (a, scored as C1) and the two squares (b,c) with only a few plaques. The middle one (b) would be scored ± and the right one (c) would be scored 1+. If there is an equal amount of lysed area and growth, the reaction would be 2+ and, if only small islands of growth appeared, the reaction would be 3+. Lysis scored as 2+, 3+, or C1 is considered a positive response.

The terminology for reporting typing results will be as follows: cultures that are sensitive to only one phage will be designated as a phage type; cultures that are sensitive to more than one phage will be designated as a phage pattern. Although we will not use the term phage group to any extent, the phages of the International Series have been divided into five groups on the basis of the similarities exhibited by phages within a group. Consequently, cultures that are sensitive to one or more phages of one group are classified according to phage groups.

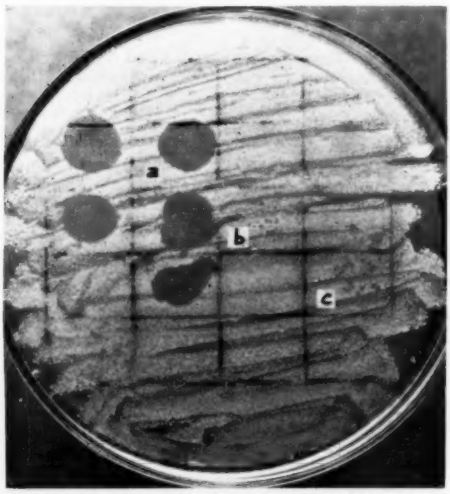

Fig. 1—A Petri dish, showing the grid marking and a culture that has been phage typed, after 14 to 18 hours of incubation at 30 C.; (a) confluent lysis, scored as C1; (b) scored ±; (c) scored 1+.

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‡ Dr. W. Thorsell, Royal Veterinary College, Stockholm, Sweden.

RESULTS

The main part of this study was on phage typing of micrococci of bovine origin with the seven phages listed (table 1). Approximately 429 cultures, 379 coagulase-positive and *beta*-hemolytic and 50 coagulase-negative cultures, were phage typed. None of the 50 coagulase-negative cultures were sensitive to the phages in use, but 354 of the 379 coagulase-positive cultures were sensitive to one or more of the phages (tables 3, 4). The phage-sensitive cultures have been divided into 12 types and patterns. Of 84 cultures from 11 herds, 78 were sensitive to only phage S2 (table 4). The remaining six cultures were not sensitive to any of the seven phages. This same phage, which is the predominant type, lysed 49 per cent of all of the sensitive cultures (tables 3, 4). Other common phage types or patterns were: S4, S6 with 16.7 per cent; S6 with 19 per cent; S5 with 7.6 per cent; S1, S3, S5 with 5.9 per cent; and S2, S5 with 4.2 per cent. These phage types and patterns account for 94.7 per cent of the phage-sensitive cultures. From tables 3 and 4, it is apparent that a common phage type or pattern generally existed among cultures from herds from which a relatively few cultures were isolated. For example, the cultures from herd O were of phage pattern S1, S3, S5; those from the herd N were of phage type S5. However, several phage types and patterns existed in herds from which a relatively large number of cultures were isolated, as shown by the cultures from herd G.

TABLE 3—The Frequency of the Phage Types and Patterns of Cultures from 12 Herds

	Phage type or pattern *												Total cultures	
Herd	42D S2	42D S2 S5	S1 S3 S5	S2	S2 S5	S2 S5 S6	S2 S4 S5 S6	S3	S3 S5	S4 S6	S5	S6	Phage-sensitive	Per herd
A	1	....	....	27	....	....	1	....	....	35	2	2	68	70
B	2	....	....	8	1	....	....	....	....	3	2	....	16	16
C	....	....	1	....	....	....	....	....	....	4	....	....	5	5
D	1	....	....	1	....	....	....	....	....	....	15	....	17	17
E	....	....	....	4	2	....	....	....	....	....	2	....	8	8
F	....	....	2	....	3	....	....	....	....	....	....	....	5	5
G	....	....	9	42	1	....	....	2	2	....	....	1	57	58
H	....	3	1	3	8	....	....	....	....	6	3	31	55	56
J	....	....	....	10	....	....	....	....	....	....	3	....	13	17
K	2	....	....	....	....	....	....	....	....	....	....	5	7	7
L	....	....	4	....	....	2	....	....	....	2	....	....	8	9
M	1	....	....	3	....	....	....	....	....	7	....	....	11	12
Per cent of total †	2.3	0.9	5.9	49.3	4.2	0.6	0.3	0.6	0.6	16.7	7.6	11.0	270	280

* At the routine test dose (RTD); † includes data given in tables 3 and 4.

TABLE 4—Herds in Which Only One Phage Type or Pattern Occurred

Herd	Phage type or pattern	Total cultures: Phage-sensitive	Total cultures: Per herd
N	S5	2	10
O	S1, S3, S5	4	5
P	S2	5	5
Q	S2	12	12
R	S2	4	4
S	S2	5	7
T	S2	10	10
U	S2	2	6
V	S2	9	9
W	S2	2	2
X	S2	22	22
Y	S2	5	5
Z	S2	2	2
Totals		84	99

In considering the phage type or pattern of cultures isolated from the same cow, but from different quarters before and after therapy, the following results were often obtained. From a quarter designated as quarter A, a phage type S2 culture was isolated. From quarter B of the same cow, a culture of phage pattern S1, S3, S5 was isolated. This cow was then treated with antibiotics and, subsequently, cultures of the same phage type and pattern as prior to treatment were isolated from each of the respective quarters—good evidence that the cultures had survived the antibiotic therapy and were not a result of reinfection following therapy. In another case, a phage-type S2 culture was repeatedly isolated from the same quarter at monthly intervals over a period of six months following antibiotic therapy. In another cow,

the pretreated udder shed micrococci of phage pattern S1, S3, S5, but after treatment the same udder shed micrococci of phage type S2, which might indicate reinfection following successful antibiotic therapy. Williams-Smith [16] reported similar findings, but none of the infected udders had been treated with antibiotics.

Because cultures of phage type S2 were so common, an attempt was made to determine if these cultures were actually the same. This was done by determining the lysogenicity of the S2 type cultures. Willams-Smith [17] subdivided the predominant 42D type cultures into nine subtypes, with the use of seven phages carried by the 42D type cultures. We tested the lysogenicity of the S2-type cultures by spotting filtrates of four-hour broth cultures, from several S2 type cultures, on plates which were inoculated with the same cultures.[5,8] Three cultures, 11, 108, and 165 (table 5), from

TABLE 5—Demonstration of Phages Carried by Three Micrococci Sensitive to Phage S2

Herd	Culture	Broth culture filtrate		
		11	108	165
P	11	—	CL*	CL
Q	108	—	—	CL
R	165	—	CL	—

* CL = Confluent lysis; — = no reaction.

different herds, were tested to determine which cultures, if any, carried phages or were lysogenic. Filtrates from cultures 108 and 165 lysed culture 11, the filtrate of culture 108 also lysed culture 165, and the filtrate of culture 165 lysed culture 108. In no instance did the filtrate of the three cultures lyse themselves, and the culture filtrate from culture 11 did not lyse cultures 108 or 165. It should be noticed that culture 11 is the propagating strain for phage S2 (table 1).

Phages carried by and isolated from cultures 108 and 165 and propagated on culture 11 are listed as phage 108/11 and phage 165/11, respectively (table 6). The cultures from P, Q, and R were then typed

TABLE 6—Subdivision of Micrococci Sensitive to Phage S2

Herd	No. of cultures	Phage		
		S2	108/11	165/11
P	5	CL*	CL	CL
Q	12	CL	—	CL
R	4	CL	CL	—

* CL = Confluent lysis; — = no reaction.

against the three phages, S2, 108/11, and 165/11.

Using the two phages, 108/11 and 165/11, it was possible to subdivide the phage type S2 cultures into three subtypes (table 6). Cultures from herd P have been designated as subtype S2, 108/11, 165/11; cultures from herd Q have been designated as subtype S2, 165/11; and cultures from herd R have been designated as subtype S2, 108/11. It is obvious that all cultures from one herd were of the same subtype, but that cultures from the three herds were each of a different subtype.

TABLE 7—Phage Type or Pattern of Five Penicillin-Resistant Micrococci

Herd	Culture	Phage type or pattern	Sensitivity to penicillin (units/ml.)
A	123	S2, S4, S5, S6	>10 <100
S	265	NT*	>10 <100
J	402	S5	>10 <100
A	423	S5	> 1 < 10
A	438	S5	>10 <100

* NT = no reaction.

Antibiotic-sensitivity tests were done on 119 of the 450 cultures that were phage typed. Of these 119 cultures, only five were found to be resistant to more than 1 unit per milliliter of penicillin (table 7). Three of the five penicillin-resistant cultures were of phage type S5, the fourth culture was of phage pattern S2, S4, S5, S6, and the fifth culture was not sensitive to any of the phages in use.

Cultures isolated from milk in Sweden were phage typed to determine whether cultures isolated in an area outside of Wisconsin were sensitive to the phages we isolated. Among 34 cultures, 25 were typed as phage S2, four were of phage pattern S4, S6, three were of phage type S5, one was of phage pattern 42D, S2, and one was not sensitive to the phages in use. In general, the frequency of the phage types and patterns among the cultures obtained from Sweden was found to be similar to those isolated from milk in Wisconsin (table 3).

Bovine Cultures Typed Against Phages of International Series, Blair Phages, and Jackson Phages.—Approximately 102 cultures were selected at random from our collection of cultures of bovine origin and typed with phages which were originally selected for the typing of micrococci of human origin. The typing results of these

cultures against the 19 phages of the International Series, the six phages from Blair, and the 12 phages from Jackson are shown (table 8). Over half, 55 of 102 cultures were lysed by phage 44A. Of these 55 cultures, 36 were lysed by phage 44A alone, and 11 were lysed by phages 42D and 44A. The other phage-sensitive cultures have been segregated into 14 phage types and patterns. Thirty-four cultures were not sensitive to any of the above phages, but 13 of the 34 were sensitive to one or more of our six phages (table 1). Phage 44A is in the miscellaneous group and phage 42D is in group IV, according to the phage group classification.[17] Thus, on that basis, most of the cultures described above fall into the miscellaneous group and into group IV type cultures; the remaining cultures fall into group III.

TABLE 8—Sensitivity of 102 Cultures of Bovine Origin Against Blair Phages, Jackson Phages, and Phages of International Series

Phage type or pattern	Frequency
VA4, 42E, 47, 53, 77	1
VA4, 42E, 47A, 54, 79, 81	1
VA4, 42E, 47A, 47B, 53, 54, 77, 79, 81	1
VA4, 53, 77, 79	1
7, 42B, 42D, 44, 44A, 54, 81, 971	1
29, 31, 44A, 52A, 971	2
29, 42D, 52A	4
31, 44, 44A	1
42B, 42D, 42E, 971	1
42D	1
42D, 44A	11
42D, 42E, 44, 44A, 47B, 54, 971	1
44A	36
44A, 52, 52A	2
54	1
79	2
NT*	34

* NT = no reaction.

DISCUSSION

Of 379 coagulase-positive micrococci that have been incriminated in bovine mastitis, 93 per cent of the cultures were sensitive (table 3, 4) to one or more of the seven phages listed (table 1). None of the 50 coagulase-negative cultures were sensitive to any of those phages. The 354 sensitive cultures represent 93 per cent of the coagulase-positive cultures. Williams-Smith, in England,[16] typed 93 per cent of the cultures that were isolated from milk, and found phage type 42D to be the most common among the cultures he tested. He used 21 phages which he obtained from Wilson and Atkinson[18] and 11 additional phages which he isolated during the course of his phage typing. Thatcher and Simon[14] found that phage type 42D was the predominant type among cultures isolated from butter and cheese in Canada. However, McLean[7] found phage type 44A to be the predominant type among cultures isolated from milk in Australia. All our cultures of phage type S2 were also sensitive to phage 44A, and approximately 80 per cent of the 44A type cultures were sensitive to phage S2.

Because we were aware of the limitations of the present typing method, an effort was made to develop a more satisfactory technique. Williams and Rippon[15] suggested two approaches for further studies, which they believed would be more reliable. The first was to search for a phage from which "adapted" phages could be prepared that would be type specific. Although the present typing method permits the designation of micrococci into phage types, they actually were not type specific in the strict sense. The second suggestion was to identify cultures according to the phages they carried. Boyd[4] used this method for identifying phage types of *Salmonella typhimurium*.

We investigated both approaches suggested by Williams and Rippon.[15] Five "adapted" phages were prepared from two phages, 42D and S3 (table 1). With the use of these phages we were unable to type specifically all of the phage-sensitive cultures, even though many of them were sensitive to only one phage (table 3, 4). However, by investigating the lysogenic nature of phage type S2 cultures, these cultures were subtyped (table 5, 6) The cultures from three herds, P, Q, and R, were shown to be identical, at least by the tests conducted to date. This suggests that reliable epizootiological studies may be conducted by the application of phage typing and by determining the lysogenic nature of cultures of the same phage type. Further support of this has been reported by Williams-Smith.[17] He showed how cultures of phage type 42D were subdivided on the basis of the phages they carried. Most of the cultures which Williams-Smith isolated were shown to be lysogenic; this has been our experience also.

SUMMARY

1) A method is described for phage typing of micrococci of bovine origin.

2) Of 379 coagulase-positive, *beta*-hemolytic micrococci from bovine mastitis, 93 per cent were sensitive to one or more of seven selected phages. The predominant phage type we found was similar to that reported from Australia, but different from that reported from England and Canada.

3) Cultures of the same phage type were subtyped by cross lysogenicity tests. This suggests that reliable epizootiological studies may be conducted by the application of phage typing and by determining the lysogenic natures of cultures of the same phage type.

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The Detection of Isopropyl Alcohol in Frozen Semen

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ONE OF THE methods of storing frozen semen consists of placing it in 2-cc., heat-sealed, polyethylene ampules, the walls of which vary from 0.018 to 0.031 inches in thickness. The ampules are then immersed in a 90 per cent isopropyl alcohol bath and the temperature is lowered to —79 C. and held there for the duration of the storage time. Since a high concentration of alcohol would be detrimental to the semen, the purpose of this experiment was to detect any alcohol which had diffused through the wall of the polyethylene ampule.

MATERIALS AND METHODS

The method described by Gibson and Blotner[3] for determining the ethyl alcohol concentration in blood and urine was used. The basis of this technique consists of separating the alcohol from the semen by precipitation of the protein, filtration, and distillation. The alcohol is oxidized by a potassium dichromate sulfuric acid solution, and the diminution in color of the dichromate solution is measured with a photoelectric colorimeter. Although isopropyl alcohol is not as easily oxidized as ethyl alcohol, the procedure used should completely oxidize any alcohol present to the ketone form. The equation to illustrate this reaction is:

$$6C_3H_7OH + 2K_2Cr_2O_7 + 8H_2SO_4 \longrightarrow 6C_3H_6O + 2Cr_2(SO_4)_3 + 2K_2SO_4 + 14H_2O$$

A survey of the literature[1, 2, 4] concerning the constituents of bovine semen and diluter reveals that nothing used has been sufficiently volatile to be carried over from the semen to the distillate. The semen used was diluted with egg yolk citrate, and glycerin was added to a concentration of 7.5 per cent.

The photoelectric colorimeter readings of the semen samples were compared with the readings of standards, which consisted of samples of alcohol in water carried through the technique in the same manner as the semen samples. The standards used were 0, 50, 75, 100, and 150 mg. of isopropyl alcohol per 100 ml. An average of 12 samples was used for each standard.

Four groups of samples were used. The first consisted of five samples of semen with a concentration of 75 mg. of isopropyl alcohol per 100 ml. These samples were used to be sure that any alcohol present in the semen would be detected. The second group, consisting of ten samples of water, 7.5 per cent glycerin, and 100 mg. of isopropyl alcohol per 100 ml., was used to be sure that the glycerin would not interfere with the determination. The third and fourth groups consisted of semen samples. The third group, used as a control, consisted of samples which had been prepared for freezing but which had never been in contact with isopropyl alcohol; 11 determinations were made on this group. The fourth group consisted of samples which had been in the isopropyl alcohol bath for periods of eight months to two years; ten determinations were made on this group.

The linear regression coefficient of colorimeter scale readings on concentration, in milligrams per 100 ml., and the confidence intervals were determined by the method of least squares as described by Ostle.[4] A coefficient of risk of 0.05 was used. The mean concentrations of alcohol in the samples were estimated from the linear regression coefficient and the mean photometer readings.

RESULTS

The confidence intervals of the semen and alcohol (75 mg./100 ml.) group and the glycerin and alcohol (100 mg./100 ml.) group included the concentration of alcohol used. The estimated mean of the semen and alcohol group was 82.5 mg./100 ml.

The confidence intervals for the controls and for the samples which had been stored in the alcohol bath included zero. The estimated mean of the controls was 5.5 mg./100 ml., and that of the semen stored in alcohol was 6.3 mg./100 ml. The confidence intervals and estimated means are illustrated (graph 1).

CONCLUSION

The estimated mean of 82.5 for the semen and alcohol group was 7.5 mg./100 ml. more than the concentration of alcohol used. This variation, along with the variation from zero of the controls and the principals, was not considered to be significant. However, it may indicate that some small amount of oxidizable material was carried over into the distillate. Since this variation was nearly the same for all three groups, and since the confidence intervals included 75 mg./100 ml. and 0 mg./100 ml., respectively, it is concluded that any oxi-

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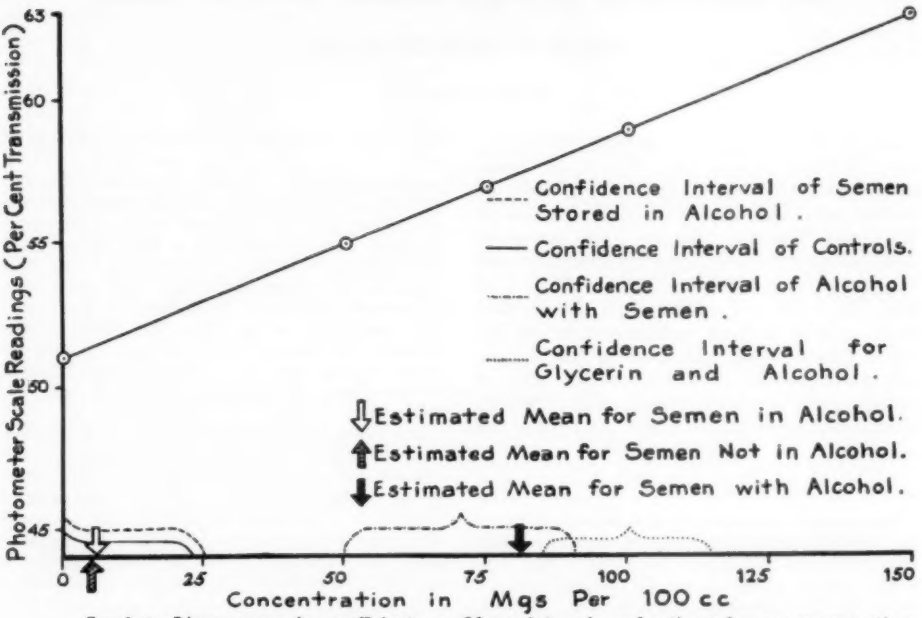

Graph 1—Linear regression coefficients, confidence intervals, and estimated mean concentrations of isopropyl alcohol in frozen semen stored in ampules and immersed in alcohol.

dizable material present was not isopropyl alcohol which had diffused into the semen during storage.

SUMMARY

Samples of frozen semen which had been stored in polyethylene ampules immersed in an isopropyl alcohol bath were used to quantitatively detect the presence or absence of any alcohol which may have diffused into the semen. The samples used had been stored in the alcohol bath for eight months to two years. It was concluded that no alcohol diffused into the semen in this period.

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A New Approach in Chemotherapeutic Trials Against Trichomonas Infections

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THERE IS no completely satisfactory treatment for a bull infected with *Trichomonas foetus*. The most frequently used treatment is Bovoflavin salbe.[1] Sodium iodide[2] and hydrogen peroxide[4] are used to a lesser extent, and the use of the first two agents in combination has been reported.[6] All available methods, however, present serious limitations concerning either efficacy or method of use.

In an attempt to develop a more satisfactory treatment for this infection, the writers have employed a method of drug screening, suggested by Kradolfer,[5] which utilizes hamsters that have been experimentally infected intravaginally with *T. foetus*.* The results obtained on 629 test animals in response to a total of 29 drugs for the period from March, 1956, to March, 1957, are reported.

MATERIALS AND METHODS

Female hamsters, usually between 2 and 3 months of age, but occasionally older, were used in these experiments. They were fed a diet of pellets† and water. These hamsters were exposed to infection by the introduction into the vagina of 0.1 ml. of a 48-hour-old culture of *T. foetus*‡ (containing an estimated 100,000 organisms), using a 1-ml. tuberculin syringe and a 14-gauge, 2-inch needle, around the blunted end of which had been placed a small ball of solder. They were examined 10 to 14 days later, and if living trichomonads were found, an infection was considered established.

Prior to these experiments, poor results had occurred in attempts to establish intravaginal infections with a strain of trichomonads that had been maintained on an artificial medium for about two and one half years. When the freshly isolated strain BP-3 was substituted, improved results were immediately evident. During the year that this strain has been used, about 85 to 95 per cent of the initially exposed hamsters have become infected.

To obtain material for examination, a mucosal scraping of the vagina was made with a flame-sterilized bacteriological loop. The material thus obtained was agitated in a drop of water and examined. Those hamsters found to be infected were isolated and subsequently given medication.

The drugs tested were selected on the basis of reported or suspected antiprotozoal activity. For the most part, these drugs were added to distilled water, usually at the rate of 100 mg. per milliliter, and introduced into the vagina, stomach and, in certain instances, by other routes. For oral medication, the previously described blunted needle was inserted through the mouth and down the esophagus. The vaginal dose generally approximated 0.1 ml. of concentrated mixtures, while systemic trials were calculated on a milligram per kilogram basis. Dosage rates were, at times, based on findings of previous toxicity tests, or on rates determined for other animals by other investigators but, when necessary, they were arbitrarily chosen. Three to five daily treatments were usually given. In a few instances, the dosage and route of administration suggested by manufacturers of certain chemicals were followed even though they differed from the usual routine. Generally, the hamsters were examined seven to ten days later to determine the efficacy of treatment. This interval was considered sufficient for the increase to a detectable level of any Trichomonas population that might have been reduced by the medication. Because spontaneous recovery probably does not occur (Kradolfer[5]) and because infections of long duration are regularly observed in unsuccessfully treated and untreated hamsters, a hamster in which no trichomonads were found after treatment was presumed to have been freed of them by the particular medication used for treatment. Since the work was of a preliminary nature, two direct examinations, on different days, which were negative for infection were considered indicative of recovery.

RESULTS

The experimental results and further information concerning the chemicals used in these experiments are tabulated. Of the 29 drugs listed, the seven apparently exhibiting a discernible degree of trichomonacidal activity were aminitrozole, antryeide methyl sulfate, Berenil, Bovoflavin, enhep-

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The writers are indebted to Dr. Louis S. Diamond, Protozoan Parasite Section, for cultures and numerous helpful suggestions.

* Reusse,[9] and Uhlenhuth and Schoenherr[10] have also reported experimental vaginal infections of hamsters with *T. foetus*.

† Animal Foundation Laboratory Diet, Standard Brands, Inc., New York, N. Y.

‡ This strain, originally isolated from a bull at Logan, Utah, in March, 1956, is maintained in this laboratory and identified as BP-3.

TABLE 1—Trichomonacidal Activity of 29 Compounds Tested in a Total of 629 Hamsters

Chemical and technical name	No. of hamsters	Route of administration *	Dosage	No. of treatments	Efficacy (%)
Acriflavine (2:8-diamino-10-methyl acridinium chloride)	3	O	15-30 mg./kg.	4	0
	13	V	5 mg.	3	15
Aminitrozole (2-acetylamino-5-nitrothiazole)	2	O	300 mg./kg. (T)	5	100
	6	O	200 mg./kg. (T)	4 or 5	88
	21	O	100 mg./kg.	4 or 5	86
	20	O	100 mg./kg.	3	55
	15	O	50 mg./kg.	3	40
	15	O	50 mg./kg.	4 or 5	80
	33	V	10 mg.	3 or 5	40
Antrycide methyl sulfate [4-amino-6-(2′-amino-6′-methyl pyrimidyl-4′-amino) quinaldine-1:1′-dimethyl sulfate]	19	SC	5-25 mg./kg.	5	16
	11	V	10 mg.	5	54
Berenil[1] (p, p′-diguanyl-diazoamine-benzene)	9	O	50-200 mg./kg. (T)	5	11
	10	V	20 mg.	2 or 3	80
Bovoflavin [2: 8-diamino-10-methylacridinium chloride + 2: 8-diaminoacridine monohydrochloride and bis-(2-methyl-4-amino-quinolyl-6)-carbamide hydrochloride]	6	V	0.1 ml.	5	67
Camoform [2] (diallyl-diethyl-amino-methyl phenol hydrochloride)	9	O	250-500 mg./kg.	4	22
	10	V	30-50 mg.	4	33
	7	V	10 mg.	3	0
Carbarsone (p-carbamino phenyl-arsonic acid)	9	O	25-200 mg./kg.	3	0
	10	V	20 mg.	3	10
Carlin K_1 [3] (ethylene diamine tetra acetate polyoxyethylene monyl phenol sodium dioctyl sulfosuccinate)	16	V	0.1 ml. of 0.1% solution	1, 2, or 3	0
Chloroquine [7-chloro-4-(4-diethylamino-1-methylbutyl amino)-quinoline diphosphate]	10	O	50-200 mg./kg.	3	0
	8	V	20 mg.	3	0
Dipterex (O, o-dimethyl-2, 2, 2-trichloro-1-hydroxy ethyl phosphonate)	6	O	200 mg./kg. (T)	3	0
	9	V	10 mg.	3	0
Endomycin with Neomycin vaginal powder	13	V	10 mg.	3 or 5	0
Enheptin (2-amino-5-nitrothiazole)	6	O	500 mg./kg. (T)	3	17
	8	O	300-400 mg./kg.	3	13
	12	V	20 mg.	3	92
Furazolidone [n-(5-nitro-2-furfurylidine)-3-amino-2-oxazolidone]	28	V	20 mg.	3 or 5	82
	12	V	10 mg.	3	57
NF-179 [n-(5-nitro-2-furfurylidine)-3-amino-5-methyl-2-oxazolidone]	9	V	10 mg.	3	11
NF-246 [n-(5-nitro-2-furfurylidine)-1-amino-2-imidazolidone]	10	V	10 mg.	3	0
NF-300 [n-(5-nitro-2-furfurylidine)-3-amino-5-methyl-2-thiooxazolidone]	12	V	10 mg.	3	75
	8	V	10 mg.	1 or 2	25
Nicarbazin (4, 4′ dinitrocarbanilide and 2-hydroxy-4, 6-dimethyl pyrimidine)	10	O	0.125% in feed	17	0
Nithiazide [1-ethyl-3(5-nitro-2-thiazolyl) urea]	8	O	100-200 mg./kg.	4	13
	9	V	10 mg.	3	0
Phenothiazine (thiodiphenylamine)	10	O	250-500 mg./kg.	5	0
	10	V	10 mg.	5	30
Piperazine adipate	18	O	20-200 mg./kg.	5	11
	17	V	20 mg.	3	0
Piperazine (chloride salts) [4] piperazine 15.83% (mixture of mono and dihydrochloric salts)	13	V	15 mg.	3	7
Polysorbate-80 (mono-oleate-polyoxyethylene sorbitan)	8	V	10 mg.	5	25
PR-445 [5] [n, n′ bis (2-methyl-6-amino-4-quinolyl)-1, 6-hexanediamine dihydrochloride]	4	O	100 mg./kg.	1	0
	4	SC	100 mg./kg.	1	0
	4	IP	100 mg./kg.	1	0
	4	IM	100 mg./kg.	1	0
	4	V	10 mg.	3	0
Promethazine [n-(2-diethylamino-2-methyl)-ethyl phenothiazine hydrochloride]	9	O	50-200 mg./kg. (T)	3 or 5	0
	15	V	5-10 mg.	3	24
Puromycin [6] (puromycin hydrochloride)	5	O	100 mg./kg. (T)	5	0
	4	V	10 mg. (T)	3	0
Pyrathiazine [n-(2-pyrrolidinoethyl)-phenothiazine]	4	O	200-300 mg./kg.	3	0
	3	V	5 mg.	3	0

Table 1 (Continued)—Trichomonacidal Activity of 29 Compounds Tested in a Total of 629 Hamsters

Pyrimethamine (2, 4-diamino-5-chlorophenyl-6-ethyl-pyrimidine)	6	O	100-500 mg./kg.	5	0
	18	V	10 mg.	5	0
Quinacrine [3-chloro-7-methoxy-9-(1-methyl-4-diethyl aminobutyl-amino)-acridine dihydrochloride]	8	O	100 mg./kg. (T)	4	0
	10	V	10 mg.	3	0
Viozene [7] (O, o-dimethyl O-2,4,5-trichlorophenyl phosphorothioate)	2	O	250 mg./kg. (T)	5	0
	5	O	100 mg./kg.	5	20
	22	V	0.1 ml. of 25% emulsion	3	4

* O = oral; V = intravaginal; SC = subcutaneous; IP = intraperitoneal; IM = intramuscular; (T) = fatally toxic.

[1] Squibb; [2] PAA-701, Parke, Davis; [3] Carlin; [4] Pig Tee, Gland-O-Lac; [5] Abbott; [6] Stylomycin, Lederle; [7] Dow.

tin, furazolidone, and NF-300. Of this group, aminitrozole seemed unique in having systemic as well as topical activity.

Certain drugs were fatally toxic (table 1). Thus, more hamsters were treated than shown, since the table accounts only for animals that survived treatment and were subsequently examined. Different rates of dosage and numbers of treatments have been tabulated as a unit when the variation of these factors did not influence the results. Drugs recommended specifically by the manufacturer for topical application (Bovoflavin salbe, Carlin K_1, Endomycin and Neomycin vaginal powder) and the nitrofurans were used only as douches. The others were tried topically and systemically. In instances where little or no effect followed intravaginal application, the same drug was also ineffective when given systemically and, with one exception, the drugs that showed some action topically failed to produce comparable results systemically.

Discussion

Employment of hamsters in experimental chemotherapy of *T. foetus* infections offers advantages over *in vitro* studies, as well as over other potentially available laboratory host-parasite systems and methods, such as mice infected subcutaneously or intraperitoneally. The vaginal mucous membrane provides a habitat more nearly resembling that of the penile or preputial mucous membrane of an infected bull than do the subcutaneous or intraperitoneal tissues of mice. However, it is yet to be demonstrated whether the chemotherapeutic effects encountered in this host-parasite relationship would be obtained in infected bulls. Preliminary studies to determine this are being made. Since topical application of medicaments to the genital membranes of bulls carries inherent difficulties, a drug, such as aminitrozole, that shows systemic efficacy warrants fuller investigation. Oral therapy with this particular drug has been shown to be effective in *Trichomonas vaginalis* of monkeys by Cuckler *et al*,[3] and of man by Plentl *et al.*[8] and Perl *et al.*[7]

Summary and Conclusions

Twenty-nine drugs were tested topically, systemically, or by both methods, against *Trichomonas foetus* in 629 vaginally infected hamsters. One drug, aminitrozole, showed promise of systemic influence on the infection. The experience, in general, indicated the usefulness of the infected female hamster for screening drugs of potential value in *T. foetus* infections.

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Observations on the Morphology of *Anaplasma Marginale* with Reference to Projections or Tails

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THIS EXPERIMENT was undertaken to screen two experimental drugs for the treatment of anaplasmosis. The observations reported were unexpected.

MATERIALS AND METHODS

For this study, 6 grade yearlings of predominantly Hereford breeding were purchased locally and splenectomized several weeks prior to inoculation. The animals were numbered consecutively, placed in groups of 2, and the groups were inoculated approximately 20 days apart.

The inoculum was secured from a normal Hereford yearling which, several months previously, had had a severe attack of anaplasmosis. This animal had been inoculated with blood from another with a virulent field infection of anaplasmosis.

At the same time, 2 splenectomized calves were each given 5 cc. of fresh whole blood from the carrier animal, subcutaneously. Temperatures were taken daily thereafter. Daily blood smears and hematocrit readings were made from the time marginal bodies were first observed until the termination of the experiment.

OBSERVATIONS

Group 1.—The incubation period (time from inoculation until bodies typical of *A. marginale* were first observed) was 21 days in both animals (A and B).

Projections, or tails, on the *A. marginale* bodies were seen only on the first day these bodies appeared in the blood.

Group 2.—The incubation period was 19 days in animal C.

The projections, or tails, were first observed on the twenty-fourth day after inoculation, with approximately 1 per cent of the cells having typical *A. marginale* bodies. The tails were also observed on each of the three following days, after which time tails were not found.

Animal D died from drug treatment on the twenty-second day after inoculation, when it was beginning to show a 1 per cent Anaplasma infection. No tails were observed.

Group 3.—The incubation period was 20 days in each animal (E and F).

The projections, or tails, were first observed on the twenty-seventh day after inoculation in animal E, at which time approximately 18 per cent of the erythrocytes showed marginal bodies. One tail was observed on the following day, but none were found afterward. This animal died four days later. Approximately 30 per cent of the erythrocytes showed marginal bodies, and the animal's hematocrit reading was 8 at the time of death.

The interval from the first appearance of Anaplasma bodies until the animals died ranged from nine to 15 days.

DISCUSSION

In Dikmans' review of the literature [3] concerning the morphology of anaplasmosis, he quotes several authors as follows: (Gomes de Faria [2])—"Besides these regular rod forms, there are at times comma-like forms with a dense spherical portion with a filamentous tail which at times appears double. These forms correspond to those seen by Quevado [5] and Descaseaux.[1] Quevado, in one of his articles, considered these forms as being due to the throwing out of fine prolongations in the shape of flagella and Descaseaux mentions their occurrence in organ smears of animals dead of anaplasmosis."

We observed, in one slide, the bodies described by de Faria. However, none of the photomicrographs in Dikmans' article showed the projections we are attempting to describe in this paper.

Lotze and Yiengst [4] may have described the forms, although their photomicrographs make it difficult to definitely establish this. Also, we find it impossible to say that they are extraerythrocytic. They definitely appear to be attached to the marginal body in the cell. This observa-

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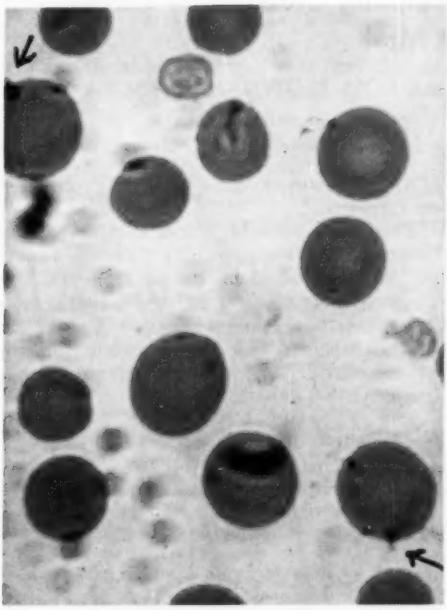

Fig. 1—Arrows indicate projections, or tails, protruding from typical Anaplasma marginale bodies in bovine erythrocytes. x 900.

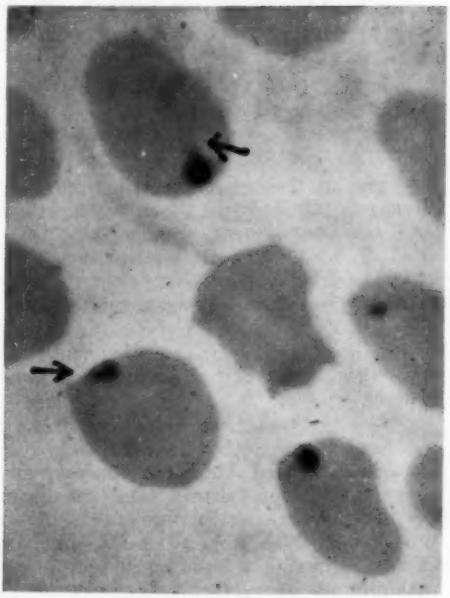

Fig. 2—Arrows indicate projections, or tails, extending into bovine erythrocytes. x 900.

tion has been confirmed by several microscopists (fig. 1, 2).

The bodies appear to be within the ordinary size and location of typical *A. marginale* (0.2 to 0.5 μ). The body stains a deep purple or magenta with Giemsa's stain, and the tail portion usually stains a light pink and extends outside of the erythrocyte. However, if the slides are stained with alkaline water for Giemsa's stain solution, the tails take a basophilic stain and are apparent. The tails vary in length, but their lengths may exceed the width of a large Anaplasma body (0.6 to 0.9 μ).

These tails have occasionally been observed in nonsplenectomized calves.

SUMMARY AND CONCLUSION

Observations were made on 6 splenectomized calves which were inoculated with blood from an animal known to be carrying anaplasmosis. Projections, or tails, extending from typical Anaplasma bodies were observed at different times in 5 animals. Similar tails have since been observed in the blood of nonsplenectomized calves that were infected with anaplasmosis. This phenomena has only been observed with this strain of the organism.

It is probable that this represents one stage in the normal development of *Anaplasma marginale* in the blood of cattle.

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Isolation of an Organism Resembling *Clostridium Carnis* from Mink

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REPORTS ON the occurrence in animals of disease attributed to *Clostridium carnis* have been infrequent. Sompolinsky [4] isolated a bacterium from diseased mink which he described as closely related to the histotoxic bacillus of gas gangrene, *Cl. carnis* (Klein) Spray. To our knowledge, the report presented here is the second such report and, for this reason, may be of some general interest.

Of 3 mink presented for examination, 1 was dead and the other 2 were killed. Case histories revealed that the disease, suspected to be leptospirosis, was enzootic on the ranch, resulting in a mortality of approximately 5 per cent. Death usually occurred following a brief period of illness without any characteristic syndrome. Although the animals were not emaciated, their coats were rough. No crusting was observed around the nasal orifice and, upon gross observation, reflex responses appeared to be unimpaired.

At necropsy, the livers of all 3 mink showed evidence of icterus, with many subcapsular petechial hemorrhages. The kidneys were swollen, and profuse petechial hemorrhages and numerous pin-point necrotic foci were observed on the subcapsular surface. Other organs appeared to be normal. Essentially, the gross pathology was the same in all 3 mink and suggested the presence of a septicemic infection.

Suspensions of the liver and spleen were placed in Verwoort's medium in an attempt to cultivate and recover any leptospiras which may have been present. Four consecutive passages were made from the original transplant. Each subculture was incubated at 32 to 35 C. for three weeks.

Spirochetes were not recovered. In addition, histological examination of liver, spleen, and kidney sections, stained with 1 per cent silver nitrate, did not reveal the presence of leptospiras. The agglutination-lysis test for leptospirosis also proved negative. Distemper virus was not recovered on ferret test.

The gross pathology was similar to that described by Sompolinsky [4] in his report on the isolation of *Cl. carnis* from mink in Denmark. Inasmuch as preliminary tests indicated that the etiological agent was not Leptospira, an examination for the presence of pathogenic anaerobes was initiated.

MATERIALS AND METHODS

A 10 per cent pooled suspension of the livers and kidneys from all the mink was made in peptone N-Z-amine broth. This material was streaked on horse blood agar plates and on desoxycholate citrate lactose sucrose (DCLS) agar plates and planted, as well, in liquid thioglycollate medium. The plates were incubated at 37 C. for 24 hours under aerobic and anaerobic conditions; the thioglycollate culture, at 37 C. for 24 hours.

In addition, 0.5 ml. from each suspension was inoculated intraperitoneally into 2, 35-Gm. hamsters, 2 suckling guinea pigs, and 5, 20- to 25-Gm. Swiss mice. All test animals died within 24 hours postinoculation, showing pronounced swelling of the abdomen and edema of the lungs. Heart blood from all dead animals was streaked on blood and DCLS plates and seeded in thioglycollate medium. These plates were incubated under aerobic and anaerobic conditions. A pooled suspension of the liver, spleen, and kidney from each of the dead animals was subjected to the same procedure.

Following incubation, the thioglycollate cultures containing the original tissue suspensions, as well as those containing suspensions from dead inoculated test animals, were heated at 80 C. for 30 minutes to destroy any vegetative bacteria which may have been present. A portion of each heated culture was transferred to additional tubes of thioglycollate medium and reincubated to obtain pure cultures of any sporogenous anaerobic bacteria which may have been present.

RESULTS

Examination of DCLS plates showed no bacterial growth. Those blood plates incubated anaerobically, containing liver and spleen suspensions from all 3 mink as well as heart blood from the dead test animals, revealed the presence of hemolytic, gram-positive bacilli with subterminal spores. Sparse growth and hemolysis were ob-

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served on plates incubated aerobically. Sporulation was not seen under aerobic conditions.

Considerable gas was seen in thioglycollate cultures after incubation at 37 C. for 24 hours. Gram's stains revealed pure cultures of large, slightly swollen, gram-positive rods, occurring singly and in pairs, with subterminal spores. Gram's stains of the peritoneal fluid and heart blood from the dead test animals gave similar results.

Since it appeared that the organisms recovered from plates and thioglycollate were similar, subcultures of the bacilli from both mediums were made in differential carbohydrate mediums for species identification, and read after incubation at 37 C. for 24 hours, with the following results: Bromcresol purple milk became acid; acid with gas was noticed in dextrose, maltose, lactose, salicin, and sucrose; hydrogen sulfide was not formed, nor was indol produced; gelatin was not liquefied and nitrates were not reduced; trehalose, raffinose, xylose, arabinose, starch, inulin, mannitol, dulcitol, sorbitol, glycerol, and inositol were not fermented. These results are in accord with reported reactions of *Cl. carnis*.

SUMMARY

An organism which conformed to descriptions of *Clostridium carnis*, in the characteristics studied, was isolated from mink from a ranch where a disease, thought to be leptospirosis, was enzootic. The bacterium was not obligately anaerobic, since delicate growth was obtained under aerobic conditions as well. It appears, therefore, that the organism in question is microaerophilic. Hemolysis was noted under both conditions.

These findings agree, essentially, with those reported by Hall and Duffett.[2] In addition, the biochemical results were similar to those published by Reed and Orr[3] and in Bergey's Manual (Breed *et al.*, 1948).[1]

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